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The Chronic Toxicity of Ion Mixtures to Freshwater Organisms: *Ceriodaphnia dubia* and *Pimephales promelas* (fathead minnow)

Katherine Anne Johnson-Couch
Clemson University, kjohnson2980@gmail.com

The chronic toxicity of ion mixtures to freshwater organisms: *Ceriodaphnia dubia* and *Pimephales promelas* (fathead minnow)

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Katherine Anne Johnson-Couch
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Accepted by:
Dr. Peter van den Hurk, Committee Chair
Dr. Stephen J. Klaine
Dr. Julia L. Brumaghim
Dr. William C. Bridges, Jr
Dr. Thomas Schwedler

ABSTRACT

Dissolved ions are natural components of aquatic systems, the concentration and composition of which are greatly controlled by the surrounding geological material present. Although naturally occurring, many anthropogenic activities, including mountaintop removal mining, road deicing practices, agricultural irrigation and coal-fired power plant effluents, can greatly increase dissolved ion concentrations, as well as alter the ionic composition of freshwater systems. An increase in dissolved ions is positively correlated with an increase in salinity and conductivity. Dissolved ions have many important physiological functions within aquatic organisms, one of which is to create electrochemical gradients within cells. These gradients are necessary for controlling water and ion movement within the organism. To establish these electrochemical gradients, freshwater organisms must balance osmotic gain and passive ion loss by utilizing active transport through a series of pumps and transporters located at the gill. If the external environment exceeds a threshold tolerable for freshwater organisms, they may reallocate more energy for ionoregulation, ultimately reducing the energy available for growth, reproduction and even survival. The overall goal of this research, therefore, was to characterize the chronic toxicity of single ions and ion mixtures to two freshwater organisms, and to investigate the key mechanisms by which they exert these chronic effects.

Reproductive effects of elevated dissolved ions were initially evaluated in *Ceriodaphnia dubia*, a small cladoceran freshwater invertebrate. Following eight-day static renewal bioassays, divalent ions (calcium, magnesium, sulfate) were determined to

have the greatest effect on *C. dubia* reproduction, while monovalent ions (sodium, chloride, bicarbonate) produced the least. Additionally, binary ion mixtures resulted in additive, less-than-additive, and greater-than-additive responses, depending on the specific ion mixtures. Seven-day static renewal bioassays were also performed utilizing *Pimephales promelas* (fathead minnow), a small vertebrate species. Similar to reproductive effects described for *C. dubia*, divalent ions resulted in the largest reduction in growth compared to monovalent ions. Furthermore, *P. promelas* demonstrated mostly additive and less-than-additive effects following binary ion mixture exposures, with sulfate reducing chloride toxicity, and calcium reducing magnesium toxicity.

A positive correlation between chloride, calcium, magnesium, and sodium EC_{50} values between *C. dubia* reproduction and *P. promelas* growth may indicate similar dissolved ion potencies between an invertebrate and vertebrate species. Although some mixture interaction differences were identified, similarities in concentration-response slopes between the two organisms may indicate similar toxicological modes-of-action. These similarities suggest that *C. dubia* reproduction may be a useful predictor of *P. promelas* growth. These results will be useful in the development of future predictive models, which can aid in establishing site-specific water quality criteria. The inclusion of a physiologically-based parameter; however, would greatly improve the accuracy and predictability of such models. For this purpose, it is imperative to identify the underlying modes-of-action for these dissolved ions.

In the present study, the toxicity of ion interactions were identified by comparing the slopes of concentration-response curves. Concentration-response curves exhibiting

similar slopes possibly indicate similar modes-of-action between contaminants. It has been suggested that effects of elevated dissolved ions on freshwater organisms may be attributed to the reallocation of energy for ionoregulatory purposes. If the ion concentration in the external environment exceeds a tolerable threshold, freshwater fish may utilize more energy in the synthesis and use of ionoregulatory essential enzymes, including ATPases and carbonic anhydrase. As such, it would be expected that an increase in enzymatic activity would occur, which would reduce the energy available for growth and reproduction. Total ATPase and carbonic anhydrase activity were measured in adult *P. promelas* gill tissue following 3- and 7-day single ion and multi-ion exposures, as well as a 7-day post-exposure recovery period. Sodium bicarbonate significantly reduced carbonic anhydrase activity, while sodium and chloride significantly increased total ATPase activity. Significant effects on total ATPase and carbonic anhydrase activity in the gills of *P. promelas* did not result from sodium sulfate exposures. This was somewhat expected, as the fish gill is impermeable to divalent anions, such as sulfate. Instead, alterations in enzymatic activity along the intestinal tract may occur, where sulfate is known to increase the permeability of sodium and chloride. This possibility presents a need for identifying changes in enzymatic activity for other ionoregulatory important tissues, such as the intestines and even kidney.

The results of this research not only add to the limited dataset regarding the chronic toxicity of elevated dissolved ions to freshwater organisms, but also further demonstrate the complex nature of ion toxicity. Differences between previously described acute ion toxicity, and the chronic toxicity established by the present study,

indicate that sub-lethal effects cannot be simply explained by their effects on survival. Although acute events, such as fish kills, are very abrupt and easily identified, chronic events should not be discounted. Reproduction and growth are important aspects that contribute to organismal fitness, and ultimately affect the success of an ecosystem. Changes in these aspects can disrupt ecosystem processes over time, and are oftentimes too subtle to take early corrective actions. For this reason, establishing water quality guidelines for elevated dissolved ions based on sub-lethal effects is critical. Overall, these results provide essential information that can help manage water quality by serving as the foundation for the development of future predictive models.

DEDICATION

I would like to dedicate this work to Dr. Stephen J. Klaine (1952 – 2016) who, through his unending guidance, support and passion, helped shape me into the scientist I am today.

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CHAPTER ONE

LITERATURE REVIEW

Dissolved Ions

Total Solids, a term encompassing dissolved, suspended, and settleable solids, are further defined by their ability to pass through a 2 μm filter. Suspended and settleable solids are large substances, including silt, algae, and plankton, and as such cannot pass through this defined filter. Dissolved solids, which can pass through a 2 μm filter, are also commonly referred to as dissolved ions. These ions can either be defined as cations, those ions possessing a positive charge including sodium (Na^{+}), calcium (Ca^{2+}), magnesium (Mg^{2+}), and potassium (K^{+}), or as anions, those possessing a negative charge such as chloride (Cl^{-}), sulfate (SO_4^{2}), carbonates (HCO_3^{-}, CO_3^{2-}), and bromide (Br^{-}) (US EPA, 1997).

The surrounding terrestrial system greatly controls the dissolved ion concentrations and ionic composition of freshwater rivers and streams due to the makeup of the geological material present (Sigee, 2005). The chemical makeup of rocks and soils is the greatest contributor to freshwater dissolved ions. Other indirect methods controlling dissolved ion concentrations and ionic composition include rainfall and anthropogenic activities (Sigee, 2005).

In North America, acid volcanic rock has the highest percent total area (53%), while the rest is comprised of carbonate rocks (22.4%), shales (18.4%), sandstones (7.3%), shield rocks (19.7%) and basalt (8.4%)(Suchet et al., 2003). Acidic volcanic rocks, or acid igneous rocks, are comprised mostly of silica (i.e. quartz) and do little to

contribute to ionic makeup of freshwater systems. In fact, most soft waters, those comprised of little calcium or magnesium, are found in areas with high acidic igneous rock. Sandstones and carbonate rocks have greater contributions to overall ion concentration within surrounding freshwater systems (Thorp and Covich, 2010).

There are many methods for measuring dissolved ion concentrations within aquatic systems. Some measurements include salinity (ppt), Total Dissolved Solids (mg/L) and conductivity (μS/cm). Salinity is simply the concentration of dissolved salts in water, and is typically <0.5ppt for freshwater systems, compared to 30ppt of saltwater (US EPA, 2006). Total Dissolved Solids (TDS) is the weight in milligrams of all ions that can pas through a 2 μm filter in a known volume of water. For freshwater systems, TDS is typically 0-1,000 mg/L for freshwater, but can be as high as 35,000 mg/L in saltwater (Barlow, 2004). Due to the electrical charges produced by each ion, water is also able to pass an electric current that is measured as conductivity. For freshwater systems, conductivity is typically between 0 and 1,300 μS/cm, while saltwater can be as high as 29,000 μS/cm (Li and Migliaccio, 2011). Simply put, as the ion concentration of a water source increases, so does the salinity, TDS and conductivity. One issue with utilizing these forms of measurements is that they are all non-specific. So, although these measurements are a good representation of the overall ion concentration, they do not indicate which ions are present and at specific concentrations.

Sources of Dissolved Ion Contamination

Low ion concentrations are primarily characteristic of freshwater systems. Many anthropogenic activities, however, can contribute to elevated dissolved ions ultimately

increasing the salinity of these freshwater systems. For example, to lower the freezing point of ice during snowstorms, many states will apply a thin layer of brine or highly saline waters to road surfaces. Consequently, the residual water and salt remaining once the snow melts will eventually runoff from roadways and into nearby aquatic systems. In the United States, it has been estimated that nearly 18.5 million tons of road salt used for deicing purposes was applied to road surfaces in 2003 alone (Kelly and Matos, 2013). Corsi et al. (2010) sampled 13 freshwater streams in Milwaukee, Wisconsin and found conductivity values as high as 30,800 μS/cm, and chloride concentrations of 11,200 mg/L. At 55% of the sites tested, chloride concentrations exceeded the U.S. EPA acute water quality criteria (860 mg/L), while 100% of sites surpassed chronic criteria (230 mg/L)(Corsi et al., 2010).

Mining operations have also been reported to increase dissolved ion concentrations of freshwater streams, as well as modify the ionic composition. One practice in particular involves mountaintop removal mining. This form of mining utilizes explosives to remove surface soils, and expose coal seams. The excess soil and rock, also referred to as overburden, is then placed adjacent to the mountain in valley fills. The mine drainage that results from these valley fills can ultimately impact nearby small freshwater streams (Griffith et al., 2012). Pond et al. (2008) reported significant increases in conductivity and dissolved ion concentrations between sites impacted by mountaintop removal effluents (n=27) and unmined streams (n=10). More specifically, reference sites had a mean conductivity of 62 μS/cm, which was significantly higher than the conductivity recorded for impacted sites (1023 μS/cm). Contributing to the

significant increase in conductivity was a significant increase bicarbonate, calcium, chloride, sodium, magnesium, and sulfate concentrations (Pond et al., 2008)(Table 1.1).

Table 1.1. The change in conductivity and specific ion concentration from an un-mined to mined site (Pond et al., 2008). Values indicate mean measurements, while parentheticals indicate the range. *p* values indicate significant differences between the un-mined and the mined sites.

	Un-Mined	**Mined**	***p***
Conductivity (µS/cm)	62 (34 – 133)	1023 (159 – 2540)	0.000
HCO_3^-	20.9 (6.1 - 35)	183 (10.7 – 501.8)	0.002
Ca^{2+}	7.5 (2.7 – 12)	137.5 (38 – 269)	0.000
Cl^-	2.8 (< 2.5 – 4)	4.6 (< 2.5 – 11)	0.022
Mg^{2+}	4.2 (2.3 – 7)	122.4 (28 – 284)	0.000
Na^+	2.4 (0.7 – 5.5)	12.6 (2.6 – 39)	0.001
SO_4^{2-}	16 (11 – 21.6)	695.5 (155 – 1520)	0.000

*All values represent data collected and statistical analysis performed by Pond et al., 2008.

Studies have demonstrated not only changes in water quality conditions, but also severe biological impairment associated with mountaintop removal mining (Pond et al., 2008; Timpano et al., 2010; Pond et al., 2014). Further studies have documented more specific effects of individual organisms, such as reproduction and growth, due to elevated dissolved ions and conductivity of impacted freshwater systems (Dwyer et al., 1992; Dickerson et al., 1996; Kennedy et al., 2005; Corsi et al., 2010; Kunz et al., 2013). These changes can ultimately lead to a change in community structure.

Conductivity versus Ionic Composition

The U.S. EPA has made great efforts in developing regulatory criteria for dissolved ions based on conductivity due to the strong positive correlation between conductivity and ionic concentration, as well as its ease of measurement in environmental settings. Because each ion produces its own current, depending on the charge and

electron mobility, it is necessary to understand the specific ions that are present in a water body (U.S. EPA, 2011). As a result, water quality criteria based on conductivity must be made on a site-specific basis. Currently, a Benchmark for Conductivity has been established for the Appalachian Region, a site that is heavily impacted by mountaintop removal mining practices. The dominant ions that make up this region include calcium, magnesium, sulfate and bicarbonate, and these ions were taken into account in the development of this benchmark.

Typically, toxicity is reported in either Lethal (LC) or Effective (EC) Concentrations. These values indicate toxicity based on the mortality of organisms exposed to a contaminant, or the sub-lethal effects such as reproduction and growth. However, in the development of the conductivity benchmark, Extirpation Concentrations (XCs) were utilized. The Extirpation Concentration was defined as "the conductivity value below which 95% of the observation of the genus occur." This correlates with a 5% mortality rate (LC_5), or disappearance of a particular genus (U.S. EPA, 2011). Although this is a very conservative approach in generating water quality criteria, previous research has suggested that as a value deviates from the median lethal or effect concentration (LC_{50}, or EC_{50}), the confidence intervals surrounding those predictions increase significantly, ultimately effecting the ability to derive accurate values (Van Gestle and Hensbergen, 1997; Shaw et al., 2006).

Additionally, conductivity is an effective estimate for total dissolved ion concentration; however, it does not specify the individual ions present within a solution. As such, conductivity-based toxicity measures would assume that all ions produce the

same toxic response. Many studies, however, have suggested that the chemical makeup of a solution is extremely important in identifying the overall solution toxicity. For example, Kunz et al. (2013) sampled three freshwater streams that were impacted by mining effluents in West Virginia. Two of these streams, identified as Winding Shoals and Boardtree, were representative of sites impacted by an alkaline mine drainage, while the ionic composition of the third stream (Upper Dempsey) was characteristic of a neutralized, or chemically treated, mine drainage. Although the ionic compositions for the three sites were different, the conductivities reported were similar (1,800 – 2,100 μS/cm). Winding Shoals and Boardtree were comprised mostly of magnesium, calcium, and sulfate, while sodium and bicarbonate were dominate in the Upper Dempsey site. Acute toxicity bioassays were performed to determine the mortality associated with each site, using reconstituted waters simulating each individual site. Of the three sites, the Upper Dempsey was the only one to result in significant reduction in *Ceriodaphnia dubia* (*C. dubia*) survival. These results suggest that toxicity is strongly dependent on the actual ions present, and not necessarily how many (Table 1.2). These results corroborate previous research that indicates measurements such as TDS or conductivity are not ideal surrogates for specific ion concentrations in evaluating ion toxicity (Kennedy et al., 2005; Mount et al., 1997; Soucek et al., 2011; Kunz et al., 2013).

Table 1.2 Water quality characteristics and specific ion concentration of three sites impacted by mining operations (Kunz et al., 2013). All ion concentrations are represented in mg/L.

	Winding Shoals	Boardtree	Upper Dempsey
Conductivity (µS/cm)	1906	2367	1813
Major Ion		**Concentration (mg/L)**	
Na+	24	12	350
Mg2+	216	260	28
Ca2+	109	241	42
K+	21	21	11
SO42-	1023	1580	640
HCO3- (as mg/L CaCO3)	99	72	279

Acute Ion Toxicity

Many sources of elevated dissolved ions in freshwater systems occur quickly, and at very high concentrations. For example, agricultural irrigation drain waters in Nevada have been reported to exhibit conductivities as high as 30,000 µS/cm, a conductivity characteristic of seawater. Exposure to such high ion concentrations, even for short durations, typically results in mortality of freshwater species. As such, great efforts have been made to ascertain the acute toxicity of these dissolved ions.

One of the largest studies was performed by Mount et al. (1997), which investigated the acute toxicity of over 2,900 ions and ion combinations to three freshwater species. The purpose of this research was to not only to gain a better understanding of major ion toxicity, but to also use these results to generate predictive models for survival. Following 48-hour *Ceriodaphnia dubia* and *Daphnia magna* and

96-hour *Pimephales promelas* toxicity bioassays, the relative order of toxicity for dissolved ions was determined to be $K^+ > HCO_3^- \approx Mg^{2+} > Cl^- > SO_4^{2-}$, with the toxicity of Na^+ and Ca^{2+} being insignificant. These results suggested that anions produced the largest toxic response, whereas cation toxicity was mostly attributed to the associated anion. However, subsequent studies indicate the potential for Ca^{2+} to influence Na^+ and Mg^{2+} toxicity, as well as Na^+ to control K^+ toxicity (Mount et al., 2016). The role of each ion to contribute to the overall toxicity of ion mixture solutions is still complex; however, significant efforts have been made to fully understand the significant effects of each ion. Doing so is imperative in not only building predictive models, but also setting appropriate water quality criteria. Additional studies have been conducted to help differentiate the interactions between ions and ion mixtures for this purpose. Soucek and Kennedy (2005) demonstrated that chloride decreased sulfate toxicity to *Hyallela azteca*, a small freshwater invertebrate. More specifically, they noted that the protective effect of chloride did not occur in lower concentrations (5 mg/L and 13 mg/L Cl^-), but instead at the higher concentrations (18 mg/L, and 36 mg/L Cl^-). Interestingly, sulfate did not seem to have the same ameliorative effect on chloride in studies utilizing *C. dubia* (Soucek et al., 2011). This suggests that different freshwater species do not respond similarly to elevated dissolved ions. Subsequent studies have examined this phenomenon further: Wang et al. (2016) demonstrated that increases in chloride concentrations (10 mg/L to 25 mg/L Cl^-) did not affect sulfate toxicity to larval *P. promelas*, a vertebrate fish species; however, small alterations in potassium concentration (1 mg/L to 3 mg/L K^+) significantly reduced sulfate toxicity.

Another important factor to note is that the hardness (due to mg/L $CaCO_3$) of an exposure medium has been demonstrated to play an important role in reducing the toxicity of elevated dissolved ions (Dwyer et al., 1992; Kennedy et al., 2003; Soucek and Kennedy, 2005; Soucek et al., 2011). Mount et al. (1997) was able to take this idea a step further, and suggested that it may not necessarily be hardness, but instead the number of cations in solution that provide this protective effect. For example, when NaCl and $CaCl_2$ were tested independently, the EC50 values estimated for Cl^- was nearly identical. Although these solutions had similar toxicities the hardness varied greatly, where the hardness of the $CaCl_2$ solution was much higher than NaCl. Furthermore, when NaCl and $CaCl_2$ were tested in combination, the resulting Cl^- toxicity was significantly reduced from that of each salt independently. The reasons behind this ameliorative effect are still largely unknown. It has been hypothesized that perhaps calcium reduces gill permeability and tightens junctions, reducing the passive diffusion of ions and water into the organism (Potts and Fleming, 1970; Soucek and Kennedy, 2005).

Chronic Ion Toxicity

Due to heightened federal regulations, many dischargers are implementing new strategies to reduce the high ion content of effluents. Although there have been significant reductions in conductivity, and as a result dissolved ion concentrations, reported conductivities are still above what is typical of freshwater systems. Conductivities and TDS measurements, associated with lower dissolved ion concentrations, have been reported to decrease reproduction and growth in freshwater species (Jop and Askew, 1994; Kennedy et al., 2005; Chapman et al., 2010; Lasier and

Hardin, 2010; Armstead et al., 2016). Decreases in reproduction and growth are typical sub-lethal responses of chronic, or long-term, exposures to contaminants. Although the effects of chronic exposures are not immediately apparent, unlike mass mortality events associated with acute exposures, their consequences within ecosystems should not be discounted. Changes in organismal health and performance can reduce population growth and fitness, factors that greatly impact the success of ecosystems. Due to the problems associated with chronic exposures, it is imperative to understand the sub-lethal toxicity associated with elevated dissolved ions, not only the acute toxicity.

The majority of chronic toxicity studies have focused on the effects of anions specifically, due to acute toxicity results that suggest anions are major contributors to toxic effects. Initially, Lasier and Hardin (2010) focused on the reproductive effects of these three ions as single components and in mixtures on *C. dubia*. Effective concentrations for a 50% decrease in reproduction (EC_{50} values) for chloride (653 mg/L), sulfate (1,252 mg/L) and bicarbonate (725 mg/L) were reported. Similar values for *C. dubia* reproduction have been reported for chloride (697 mg/L), and sulfate (1,267 mg/L)(Elphick et al., 2011*a*; Elphick et al., 2011*b*). Farag and Harper (2014) reported an EC_{20} (effective concentration that reduced reproduction by 20%) for bicarbonate as 274 mg/L, but did not report an EC_{50} value. A few other studies have reported effective concentrations for other species, including fathead minnows (*P. promelas*), white suckers (*Catostomus commersoni*), mussels (*Lampsilis siliquoidea*), rainbow trout (*Oncorhynchus mykiss*), and a midge (*Chironomus dilutus*)(Chapman et al., 2010; Farag and Harper, 2014; Wang et al., 2016).

Ameliorative effects of water hardness have also been demonstrated in chronic toxicity studies. Elphick et al. (2011*a*) reported a decrease in the EC_{50} values derived for chloride from 161 mg/L Cl^- to 700 mg/L Cl^- when hardness increased from 10 mg/L $CaCO_3$ to 320 mg/L $CaCO_3$. Similar results were also reported for sulfate (EC_{50}: 465 mg/L SO_4^{2-} at hardness of 40 mg/L $CaCO_3$; EC_{50}: 1257 mg/L SO_4^{2-} at a hardness of 160 mg/L $CaCO_3$)(Elphick et al., 2011*b*). The effects of hardness on chronic ion toxicity have not been extensively studied, and data have not been extrapolated to the degree at which acute toxicity has, including differences in sources of hardness (Mg^{2+} or Ca^{2+}) or multiple cation effects.

Although elucidating the toxicity of a single ion is the initial step in understanding differences between ion effects, it is not necessarily environmentally relevant. Ions do not occur as single entities within environmental settings. Instead, ions occur in mixtures, and the ionic composition of those mixtures can shift greatly due to anthropogenic activities. As such, it would also be necessary to identify multi-ion toxicities, and better yet, examine how the toxicity of one ion can influence the toxicity of another. Very few chronic ion mixture studies have been performed to date. One such study investigated reproductive effects to *C. dubia* chloride, sulfate, and bicarbonate mixtures in three water types (low hardness/low alkalinity, low hardness/moderate alkalinity, and moderate hardness/moderate alkalinity)(Lasier and Hardin, 2010). Concentrations utilized for each ion included their respective EC_6, EC_{12}, and EC_{25} values to create a full factorial experimental design. The results of this study indicated that these

ions react in an additive manner, meaning the presence of one ion does not impact the toxicity of the second (Lasier and Hardin, 2010).

Due to the limited data regarding the chronic toxicity of dissolved ions, it is imperative that additional work be completed to understand how these ions can impact organisms on a sub-lethal basis, especially with regards to ion mixtures. These results would make significant additions to the development of future water quality criteria.

Ionoregulation in Freshwater Organisms

The toxicity of these dissolved ions may be best explained by the ionoregulatory capacity of freshwater organisms. The osmolarity, or the amount of solute particles per liter of solution, in freshwater systems is typically <25 mOsm, compared to saltwater which roughly 1,000 mOsm (Hunter and Rudy Jr, 1975; Kefford et al., 2016). Aquatic organisms, both freshwater and saltwater, maintain an osmolarity of about 300 mOsm (Pelster, 2008). Because freshwater organisms maintain an osmolarity much higher than their surrounding media, they are considered hypertonic to their external environment. This means they must have strategic mechanisms to combat the passive loss of ions and gain of water. For this purpose, freshwater organisms must expend energy in the form of active transport systems. It is believed that this energy is derived from mitochondrion-rich (MR) cells, which are equipped to handle the energy load needed for ionoregulation (Perry and Fryer, 1997). In fact, it has been estimated that rainbow trout (*Oncorhynchus mykiss*) utilize 1.5% of their resting metabolic rate for ionoregulatory purposes in freshwater, and this rate decreases to 0.5% when in seawater (Eddy, 1982). Krogh (1938) was the first to suggest ionic uptake as the mechanism controlling this homeostatic

balance. Since then, extensive research has been conducted to investigate the specific mechanisms occurring within the gills of fish, and how they interact.

Following extensive studies utilizing goldfish (*Carassius auratus*), Krogh (1938) discovered that sodium and chloride were taken up independently from one another. This work also suggested that, due to laws of electroneutrality, these fish must utilize another set of ions to initiate the uptake of sodium and chloride. It was suggested that these ions might be byproducts of CO_2, namely H^+ and HCO_3^-, and also serve as a means for waste excretion. To this day, the hypothesis suggested by Krogh (1938) is still believed to be the main mechanisms for ion uptake. Additional studies have suggested that a passive uptake of sodium in exchange for H^+ could not happen due to the laws of thermodynamics, further evidence of the need for energy during ion exchange and uptake (Avella and Bornancin, 1989; Kirschner, 2004).

Current freshwater fish gill models denote two distinct MR cell subtypes located within the gills of fish, often referred to as peanut lectin-insensitive (PNA) cells. The first, identified as the acid-secreting cell (PNA-), is believed to be the site for Na^+/H^+ exchange, whereas Cl^-/HCO_3^- exchange occurs at the base-secreting cell (PNA+)(Figure 1.1)(Marshall and Grosell, 2005). These models further demonstrate the presence of a V-type H^+-ATPase enzyme within the apical membrane of acid-secreting cells, which provides a source of energy for Na^+ uptake through apical channels. More specifically, energy (ATP) is initially required to excrete H^+ outside the MR cytoplasm. The excretion of H^+ creates an electrochemical gradient, which encourages Na^+ diffusion in the cytoplasm through sodium channels (Avella and Bornancin, 1989). The possibility for

such an intricate system was further evidenced by studies that revealed a decrease in sodium uptake during inhibition of the V-type ATPase inhibitor, bafilomycin A (Bury and Wood, 1999; Fenwick et al., 1999; Perry et al., 2003). Movement of sodium through the basolateral membrane of MR cells into the plasma of freshwater fish is believed to be primarily by Na^+/K^+-ATPase enzymes. These enzymes function by hydrolyzing one ATP molecule for every 3 Na^+ ions transferred externally and 2 K^+ ions moved internally, relative to the cell. This provides a net movement of 1+ charge, creating an internal negative charge and an external positive charge. These enzymes are located throughout the body, occurring more prominently in the gills, kidney, intestines, Red Blood Cells (RBCs), and muscle and nerve cells (Duhl and Hokin, 1974). This charge difference drives the movement of many macromolecules, such as glucose and amino acids, as well other biologically important molecules, such as oxygen (Thomas and Egee, 1998).

Although extensive research suggests that chloride is most likely exchanged for bicarbonate on the apical membrane of freshwater fish, it is still largely unknown how this mechanism properly functions due to electrochemical gradients (Perry et al., 2003). The presence of a V-type ATPase on the basolateral membrane has been suggested to provide the electrochemical gradient needed for this exchange (Piermarini and Evans, 2001). Carbonic anhydrase, a member of the zinc metalloenzyme family, is responsible for providing the bicarbonate ion needed for its 1:1 exchange with chloride. It does so by catalyzing the reversible hydration and dehydration reactions of carbon dioxide, a byproduct of respiration. During the hydration process, one ATP molecule is consumed

to transform carbon dioxide into bicarbonate and hydrogen (H^+), the H^+ ion being further excreted through the apically located V-type H^+-ATPase pump.

The kidney, as well as the intestine plays a supporting role to the gills in ionoregulation and ion uptake. The kidney in particular is important in this process for freshwater fish. In fact, approximately 95% of sodium chloride is reabsorbed through the glomerulus (Perry et al., 2003). Additionally, bicarbonate is also highly regulated at the kidney to help maintain proper blood pH. The exact mechanisms for the reabsorption of Na^+ and Cl^-, as well as the regulation of HCO_3^- within the kidney are still mostly unknown. However, similarities between the gill and kidney have been made in terms of a Na^+/H^+ interaction, Na^+/K^+-ATPase, and Cl^-/HCO_3^- exchange. Furthermore, evidence of a V-type ATPase initiating reabsorption of HCO_3^-, similar to the gill, has been demonstrated in the kidney (Perry and Fryer, 1997). Due to the reabsorption of nearly 100% of sodium and chloride, as well as the passive gain of water, freshwater organisms typically produce large volumes of extremely dilute urine (Perry et al., 2003). The intestine, although not as significant as the kidney, is also responsible for the uptake of ions. Calcium uptake is particularly important along the intestinal tissue of freshwater fish.

Euryhaline fish are those capable of moving between freshwater and saltwater. A 20% increase in the osmolarity of euryhaline fish plasma has been recorded during this transition (Bone and Moore, 2008). Furthermore, the mummichog (*Fundulus heteroclitus*, another euryhaline species, has been reported to rapidly increase Na^+/K^+-ATPase activity within gill tissue following exposure to seawater. Because these fish

naturally move between the two environments, they are better adapted to shifts in salinity or increases in dissolved ion concentrations than freshwater fish that are limited by their ionoregulatory capacity.

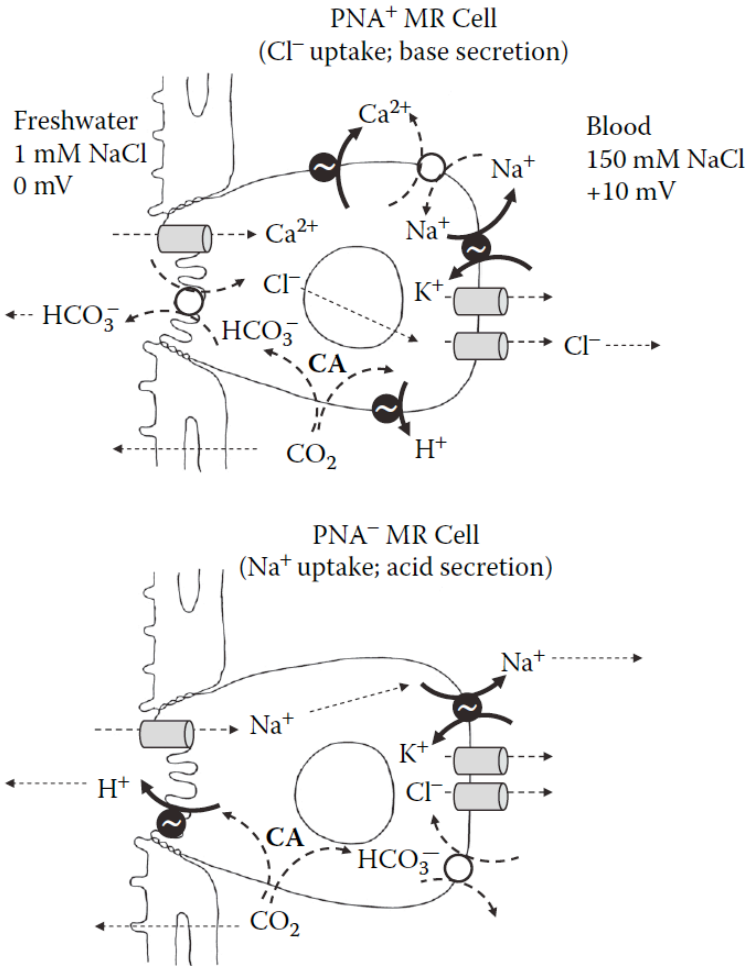

Figure 1.1. A schematic of a freshwater teleost gill. Dark circles represent ATPase pumps, gray cylinders indicate ion channels, and open circles indicate ion exchangers (Marshall and Grosell, 2005).

Overall Research Goal

The overall goal of this research was to gain a better understanding of the chronic toxicity of ions and multi-ion mixture exposures to freshwater organisms. To achieve this goal, I completed four objectives:

1. Characterize the chronic toxicity of elevated dissolved ions, both as single ions and in binary mixtures, to an invertebrate species (*Ceriodaphnia dubia*) using reproduction as the endpoint.
2. Characterize the chronic toxicity of elevated dissolved ions, both as single ions and in binary mixtures, to a vertebrate species (*Pimephales promelas*) using growth as the endpoint.
3. Compare the effects from *C. dubia* reproduction to *P. promelas* growth to determine if the toxicity of one species could be a useful predictor for another species.
4. Investigate the mechanism-of-action for single ions and ion mixtures by measuring changes in enzyme activity essential to ionoregulation.

My first and second objectives were conducted to contribute to the limited dataset describing the chronic toxicity of ions and ion mixtures to freshwater organisms. Next, I conducted a comparison of the results obtained from objectives one and two to compare between the two species tested. More specifically, I wanted to determine if the toxicity demonstrated by one species could be used to predict the toxicity of the second species, and to describe the potential for the development of predictive models based on these findings. Lastly, I wanted to gain some understanding of the mechanisms that contribute

to ion toxicity within the gill of freshwater fish, and potentially provide a physiologically based parameter that would be important for future predictive model development.

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CHAPTER TWO

THE CHRONIC TOXICITY OF MAJOR IONS AND ION BINARY MIXTURES TO *Ceriodaphnia dubia*

Introduction

Dissolved ions are naturally occurring compounds in aquatic systems that influence salinity and are frequently measured as either Total Dissolved Solids (TDS) or conductivity. Total Dissolved Solids is defined as the weight of all ions in a given solution, both organic and inorganic, that can pass through a 2 μm filter (U.S. EPA, 1997). Conversely, conductivity measures the electrical current that results from cationic (e.g. Ca^{2+}, Na^{+}, Mg^{2+}, K^{+}) and anionic (e.g. PO_4^{3-}, Cl^{-}, HCO_3^{-}, SO_4^{2-}) ion interactions. Typically, both conductivity and TDS measurements for freshwater systems are extremely low, but can easily be influenced by anthropogenic activities and disruption of surrounding geological materials. Anthropogenic activities, such as mountaintop-removal mining, agricultural irrigation, road salt application and coal-fired power plant effluents have all been reported to increase dissolved ions of freshwater systems. For example, Pond et al., (2008) reported a significant increase (p value < 0.05) in conductivity between reference sites (average 63 μS/cm; n=10) and those impacted by surface coal mining operations (average 1023 μS/cm; n=27). Previous research also suggests that an increase in dissolved ion concentration and change in ionic composition can lead to impaired organismal health and even changes in community structure (Dickerson et al., 1996; Tietge et al., 1997; Chapman et al., 2000; Kennedy et al., 2003; Pond et al., 2008; Kennedy et al., 2005). Furthermore, previous studies also suggest that

utilizing measurements such as conductivity and TDS may not be the most accurate approach to explain dissolved ion toxicity (Mount et al., 1997; Goodfellow et al., 2000; Soucek et al., 2005; Kunz et al., 2013; Mount et al., 2016)

The majority of ion toxicity data published has focused on the acute toxicity of individual ions or multi-ion mixtures (Mount et al., 1997; Soucek and Kennedy, 2005; Soucek et al., 2010; Mount et al., 2016; Erickson et al., 2017; Erickson et al., 2018). However, few studies have reported the implications for chronic exposures (Lasier and Hardin, 2010; Elphick et al., 2011*a*; Elphick et al., 2011*b*; Farag and Harper, 2014). Understanding both the acute and chronic toxicity of dissolved ions is imperative for setting discharge regulations, developing federal water quality criteria, which are essential for managing the release of ions of both point (e.g. coal fired power plant effluents) and nonpoint (e.g. runoff from roadway salt application) sources.

The chronic toxicity of major anions (HCO_3^-, SO_4^{2-}, Cl^-) to *Ceriodaphnia dubia* (*C. dubia*) as single ions, and in binary mixtures has previously been reported (Johnson, 2014). To compliment this previous research, and to gain a full understanding of the chronic toxicity of major ions to a freshwater invertebrate species, the goal of this research was to characterize the effect of major cations (Na^+, Ca^{2+}, Mg^{2+}) on *Ceriodaphnia dubia* reproduction. Additionally, results described by Johnson, 2014 were evaluated further.

Materials and Methods

Ceriodaphnia dubia Culture Methods

Rearing and testing of *C. dubia* followed the U.S. EPA guidelines for short-term methods for estimating chronic toxicity (U.S. EPA, 2002), as well as the framework previously described in Johnson, 2014. Briefly, organisms were cultured in-house at the Clemson University Institute of Environmental Toxicology (CU-ENTOX). Reconstituted moderately hard water was prepared according to U.S. EPA guidelines (U.S. EPA, 2002) with reagent grade chemicals (96 mg/L $NaHCO_3$, 60 mg/L $CaSO_4 \bullet 2H_20$, 60 mg/L $MgSO_4$, and 4.0 mg/L KCl). To aid in reproduction, the reconstituted moderately hard water was spiked with 2 µg/L selenium (as Na_2SeO_4).

Individual cultures were initiated in order to assess organismal health by separating neonates into individual 30 mL culture chambers containing 15 mL of culture media (reconstituted moderately hard water and 2 µg/L selenium), and kept in an incubator set at 25°C (±1°C) with a 16:8 light/dark cycle. Daily water renewals were performed and reproduction/mortality were recorded. Organisms were fed a diet of 1:1 *Pseudokirchneriella subcapitata* (~3.0 x 10^7 cells/mL) and YCT (yeast, trout chow, and CEROPHYLL®). Both algae and YCT were prepared at CU-ENTOX using methods described by the U.S. EPA (2002). Test organisms were selected from successful adults producing ≥15 neonates across 3-broods within a 7-day period.

Test Solutions

Test solutions were prepared using ACS grade chloride-salts of sodium, magnesium, and calcium. Sodium chloride (CAS 7647-14-5; Fisher Scientific, Atlanta,

GA), calcium chloride dihydrate (CAS 7757-82-6; Fisher Scientific, Atlanta, GA), and magnesium chloride hexahydrate (CAS 144-55-8; Fisher Scientific, Atlanta, GA) stock solutions were created using the same reconstituted moderately hard water recipe as the culture medium. Test solutions were created 24-hours prior to use by adding the appropriate volume of each salt stock solution to culture media for all bioassays. All test solutions were spiked with 2 μg/L selenium. Once prepared, test solutions were allowed to come to equilibrium in the same temperature controlled test chamber as the test organisms (25 ±1°C) to ensure no temperature changes between water changes. A sample of each test solution and controls were collected for ion analysis via ICP-MS.

Bioassay Procedure and Experimental Design

Bioassays procedures and chronic toxicity were assessed by following the basic framework of the short-term methods for chronic toxicity estimation guidelines described by the U.S. EPA (2002), with modifications. Similar to the individual cultures, one *C. dubia* neonate produced by successful culture adults was placed in a 30 mL plastic test chamber containing 15 mL of test solution at the start of each bioassay. Water renewals occurred every 24-hours over the eight-day exposure, at which time reproduction and mortality were recorded. Organisms were fed 250 μL a 1:1 mix of *Pseudokirchneriella subcapitata* and YCT immediately following water renewals. Water quality parameters, including temperature (°C), DO (mg/L), conductivity (μS/cm), pH, alkalinity (as mg/L $CaCO_3$) and hardness (as mg/L $CaCO_3$) were monitored for the initial test solution, and after 24-hours of exposure. Results for each bioassay were only accepted if control

treatments 60% of individuals produced ≥ 15 neonates across 3 broods and maintained 80% survival over the eight-day exposure.

Briefly, bioassays consisted of six treatments with ten replicates per treatment. Treatments were either single-cation exposures, or cation binary mixtures. Initially, single-cation exposures were conducted to estimate Effective Concentrations (EC) values for decreased *C. dubia* reproduction for use in binary mixture bioassays (EC_{10}, EC_{30}, EC_{50} and EC_{70}). The experimental design utilized in this approach for cation binary mixtures followed the titration method described by Johnson (2014) and Meyer et al., (2015) with modifications. This type of approach quantitatively evaluates the toxic response of one titrated ion (Ion B), when combined with a fixed concentration of another ion (Ion A) (Figure 2.1). A negative control treatment, only containing reconstituted moderately hard water, was used to assess the success of each binary mixture, as well as to standardize reproduction in all exposure treatments. A positive control treatment was also established and only contained the fixed concentration of Ion A. One cation-only bioassay was performed in conjunction with two binary mixture bioassays. The cation-only bioassay contained the same cation as the titrated cation present in the binary mixtures. For example, a sodium-only bioassay was performed with titrated sodium with fixed magnesium and titrated sodium with fixed calcium. This means that at any given time, three bioassays were being performed concurrently. The two binary mixture bioassays were then compared back to the cation-only bioassay that was simultaneously performed. Cation concentrations utilized were the same for the cation-only and binary mixture treatments.

Chemical Analysis

Samples were obtained for each test solution prior to introduction of organism (initial) and 24-hour after exposure (final) everyday of the eight-day bioassay. Water samples were filtered using a 0.45 μm filter to remove any food particles or debris then transferred to a 15 mL plastic tube. Proper dilutions were performed using a 3% HNO_3 solution with nanopure water using Trace-Grade HNO_3. Sodium, calcium and magnesium concentrations were identified using an ICP-MS at the CU-ENTOX facility. Chloride concentration, the associated anion to the three cations tested, was derived from the measured cation concentrations.

Statistical Analysis

JMP® 11.0.0 was utilized to perform all statistical analyses. For all bioassays, percent reproduction was calculated by dividing the total number of neonates produced per replicate by the average neonates produced in the negative control treatment (the treatment only containing moderately hard water) for their corresponding bioassay. Both linear regressions and Hill equations (Logistic 4P) were applied to each cation-only and cation binary mixture dataset to determine the best-fit curve. The best-fit curve was characterized as the curve having the largest R^2 value. Polynomial fits were only used for graphical representation in figures, and not for EC value estimations or slope.

Inverse interpolation was then performed on each cation-only best-fit curve to estimate the Effective Concentration at which a 50% decrease in reproduction occurred (EC_{50}) with 95% confidence intervals (α=0.05). For each cation-only dataset, both conductivity (μS/cm) and specific ion concentration (mM) were used to estimate EC_{50}

values. Significant differences between each estimated EC_{50} value was determined based on overlapping 95% confidence intervals.

Using an Analysis of Covariance (ANCOVA), significant differences between the slope of the best-fit curve for the cation-only concentration-response line, and the best-fit curve for the cation binary mixture concentration-response line were verified. Less-than-additive interactions were concluded if the slope of the mixture concentration-response line was statistically shallower than the corresponding cation-only concentration-response line. Alternatively, if the mixture slope was statistically steeper than the corresponding cation-only slope, a greater-than-additive interaction was concluded. No significant differences in slope resulted in additive toxicity (Figure 2.2).

This same analysis was also employed to data collected by Johnson (2014) in order to better characterize the chronic toxicity of anions and anion binary mixtures to *C. dubia*. Results are reported below.

Results

Ion-Only Toxicity

Estimates for a 50% decrease in reproduction for *C. dubia* were calculated from both overall conductivity measurements (µS/cm) and specific ion concentrations (mM) (Table 2.1). The order of toxicity, as described by EC_{50} values estimated from total conductivity, was $HCO_3^- \geq Mg^{2+} > Ca^{2+} > Na^+ \geq Cl^- > SO_4^{2-}$. This series indicates that some EC_{50} values based on conductivity were significantly different from one another. For example, bicarbonate[a] was similar to magnesium[a], but not calcium[b], while sodium[c] and chloride[c] were similar, and sulfate[d] was statistically different. Alternatively, the

order of toxicity, as described by EC_{50} values estimated from specific ion concentrations, was $Ca^{2+} \geq Mg^{2+} \geq SO_4^{2-} > HCO_3^- > Cl^- > Na^+$. Although no particular categorization was indicated by conductivity, the order of toxicity based on ion concentration indicated that divalent ions, such as calcium, magnesium, and sulfate, were the most toxic of the six ions tested, while monovalent ions, including chloride, bicarbonate, and sodium were less toxic.

For anion-only exposures, the bicarbonate concentration-response curve exhibited the sharpest slope –14.9 (R^2: 0.746), followed by sulfate (slope: –4.94; R^2: 0.862). Chloride had the shallowest slope of the three concentration-response curves at –3.08 (R^2: 0.739). The three anion-only concentration-response curves were statistically different (*p* value < 0.0001) (Figure 2.3).

The concentration-response curve for calcium (slope: –5.34; R^2: 0.855) was statistically similar when compared to both magnesium (slope: –7.07; R^2: 0.859) (*p* value: 0.0732), as well as sodium (slope: –4.89; R^2: 0.766) (*p* value: 0.6821). Additionally, magnesium and sodium also possessed similar concentration-response slopes (*p* value: 0.1065) (Figure 2.4).

Anion Binary Mixture Toxicity

Anion binary mixtures containing titrated chloride with a fixed concentration of bicarbonate resulted in a steeper slope than that of the chloride-only concentration-response curve, suggesting a greater-than-additive response between these two ions (*p* value: 0.0035; Figure 2.5). Conversely, when titrated chloride was in the presence of a

fixed concentration of sulfate, a significantly shallower slope resulted, indicating a less-than-additive interaction (*p* value: 0.0332; Figure 2.5) (Table 2.2).

Concentration-response curves produced for mixtures containing titrated sulfate were not significantly different from the corresponding sulfate-only concentration-response curve, indicating additive interactions when combined with a fixed concentration of both chloride (*p* value: 0.0568; Figure 2.6) and bicarbonate (*p* value: 0.701; Figure 2.6) (Table 2.2). Furthermore, the concentration-response slope of sulfate with a fixed concentration of chloride was not significantly different from the concentration-response slope of sulfate in the presence of bicarbonate (*p* value: 0.714). It should also be noted that the *p* value calculated for the sulfate concentration-response curve with a fixed concentration of chloride was close to being statistically significant, suggesting the potential for a greater-than-additive interaction between these two ions.

Both binary mixture concentration-response curves containing titrated bicarbonate resulted in less-than-additive interactions when combined with chloride (*p* value: 0.0067; Figure 2.7) and sulfate (*p* value: 0.0103; Figure 2.7), as compared to the bicarbonate-only slope (Table 2.2). There was no significant difference between the slope of the concentration-response curve for titrated bicarbonate combined with fixed chloride or sulfate (*p* value: 0.3677).

Cation Binary Mixture Toxicity

The majority of cation binary mixture concentration-response curves resulted in additive interactions, when compared back to their cation-only counterpart. More specifically, concentration-response curves produced for mixtures containing titrated

calcium were not significantly different from the corresponding calcium-only concentration-response curve, indicating additive interactions with both sodium (*p* value: 0.292; Figure 2.9) and magnesium (*p* value: 0.125; Figure 2.9) (Table 2.3). Similar additive results occurred between the sodium-only concentration-response curve and that produced for sodium in the presence of 4.27 mM magnesium (*p* value: 0.820; Figure 2.10) and in the presence of 3.26 mM calcium (*p* value: 0.660; Figure 2.10). Titrated magnesium, combined with a fixed concentration of sodium, also resulted in an additive interaction (*p* value: 0.501; Figure 2.8). Alternatively, when titrated magnesium was in the presence of 3.45 mM calcium, a less-than-additive interaction occurred (*p* value: 0.0043; Figure 2.8).

Discussion

Dissolved ions in freshwater systems have been measured at elevated concentrations due to many anthropogenic activities (Kunz et al., 2013; Pond et al., 2008; Timpano et al., 2010). Due to the positive correlation between conductivity and ion concentration, as well as its ease of measurement, it would be ideal to build water quality criteria based on toxicity resulting from overall conductivity. Because conductivity combines all ions into one parameter, it would assume that each ion has the same effect on biological systems. As a result, the same toxic effect would be expected regardless of ion or ionic composition. Reproductive effects, described by EC_{50} values derived from conductivity in the present study, resulted in significant differences between individual ions. These significant differences suggest that each ion produces the same effect, 50% decrease in reproduction, but at varying conductivities. Bicarbonate was the most toxic

with an EC_{50} value of 1322 µS/cm, while sulfate, with an EC_{50} of 2514 µS/cm, was the least toxic. These results further support previous findings that measurements, such as conductivity, are limited in their application (Mount et al., 1997; Kennedy et al., 2005; Kunz et al., 2013; Mount et al., 2016).

Anion-only bioassays utilized sodium-salts of chloride, bicarbonate and sulfate to characterize the toxicity of the associated anion (Johnson, 2014). Sodium was used as the standard cation due to its minor contribution to toxicity, unlike calcium, magnesium, or potassium (Mount et al., 1997; Lasier and Hardin, 2010; Elphick et al., 2011*a*; Elphick et al., 2011*b*). Point estimates (EC_{50}) derived from specific ion concentrations for chloride-only, bicarbonate-only, and sulfate-only bioassays were similar to previously published values (Lasier and Hardin, 2010; Elphick et al., 2011*a*; Elphick et al., 2011*b*). However, comparisons for calcium and magnesium point estimates could not be completed because previous EC_{50} values for these individual ions were not found in the published literature.

Additionally, point estimates calculated in the present study suggest that divalent ions (calcium, magnesium, sulfate) had the largest effect on *C. dubia* reproduction, more so than monovalent ions (chloride, bicarbonate, sodium). In this regard, it seems that the associated cation (sodium) or anion (chloride) did not affect the relative order of toxicity, further suggesting that both sodium and chloride contribute less, than their counterparts, to overall toxicity. Previous studies have demonstrated that elevated calcium concentrations can reduce gill tissue permeability to both ions and water, particularly in saltwater acclimated organisms (Potts and Fleming, 1970; Eddy FB, 1975; Lucu and Flik, 1999; Pic and Maetz, 1981). More specifically, calcium reduces passive sodium loss by

blocking paracellular tight junctions (Grosell et al., 2002). Soucek et al. (2011) suggested that the same mechanism by calcium, and to a lesser extent magnesium, might occur in freshwater organisms. However, these same divalent ions are known to increase intestinal uptake of chloride and sodium, which could result in a divalent ion dependent toxicity.

Bicarbonate-only exposures, unlike sulfate and chloride-only exposures, experienced a high mortality rate. Previous studies have generated an estimated 50% decrease in *C. dubia* survival (48-hour LC_{50}) between 699.0 and 740.0 mg/L HCO_3^- (Mount et al., 1997; Harper et al., 2014). The present study estimated an EC_{50} value similar to the published LC_{50} values, indicating that the mechanism by which bicarbonate exerts its toxic effect might be the same as that for reducing survival and reproduction in *C. dubia*.

Results of the anion binary mixture bioassays indicate that the only mixture resulting in a greater-than-additive effect was fixed bicarbonate with titrated chloride. This suggests that the addition of bicarbonate to chloride-dominated waters increases its effect on *C. dubia* reproduction. However, the reverse combination (fixed chloride with titrated bicarbonate) resulted in a less-than-additive effect, indicating that the addition of chloride to bicarbonate-dominated waters reduces the overall toxicity. Because sodium-salts were utilized in all anion-only bioassays, the addition of sodium bicarbonate and sodium chloride to these mixtures nearly doubled the concentration of sodium. The increase in sodium concentration, however, is most likely not a major component of the mixture toxicity. This is further explained by the decrease in reproduction for each

concentration-response curve relative to the sodium concentration. Fixed bicarbonate with titrated chloride, at 24 mM sodium, the percent reproduction was 13%; however, for fixed chloride with titrated bicarbonate, percent reproduction was 8% at 28 mM sodium. It would be expected that if sodium was the overall cause of toxicity for these mixtures, that the higher sodium concentration would produce a greater effect on reproduction. Currently, the exact mechanism by which chloride and bicarbonate gain entrance into freshwater invertebrate species is unknown; however, it is thought that an increase in external osmolality leads to a fairly proportional increase in internal osmolality. Some studies have suggested that amino acid metabolism in freshwater invertebrates can affect their ability to ionoregulate properly, particularly with regards to chloride. If the extracellular concentration of chloride shifts beyond what is physiologically capable for the organism, decreases in reproduction and/or death will occur (Greenaway P, 1979).

The addition of sulfate at a fixed concentration of 7.84 mM, to chloride and bicarbonate, resulted in a less-than-additive effect. This effect has been previously noted with regards to acute ion toxicity. Although in *Hyalella azteca*, Soucek and Kennedy (2005) demonstrated that the toxicity of sulfate, fixed at 2,846 mg SO_4^2/L across treatments, decreased with an increasing concentration of chloride. These results indicate that for both sub-lethal and acute ion mixtures, the addition of an increasing concentration of chloride results in decreased sulfate toxicity. Interestingly, mixtures containing fixed chloride with titrated sulfate resulted in additive interactions, indicating that sulfate does not have the same ameliorative effect on chloride toxicity. The same additive response was further demonstrated in acute toxicity studies (Soucek et al. 2011).

Data regarding the amelioration in toxicity of fixed sulfate with titrated bicarbonate is fairly limited. Although not explicitly stated, it appears that based on published LC_{50} values, Mount et al. (1997) did not see any toxicity amelioration in Na_2SO_4 (LC_{50} = 3080 mg/L) and $NaHCO_3$ (LC_{50} =1080 mg/L) mixtures (LC_{50} = 2630 mg/L).

Although extensively investigated with regards to invertebrates and acute ion toxicity, the ameliorative effect of calcium on sodium-dominated waters was not illustrated in the present study (fixed calcium with titrated sodium) (Dwyer et al., 1992;). More specifically, Kennedy et al. (2005) reported that *C. dubia* mortality was significantly reduced up to a 1:20 ratio of Ca^{2+}:Na^{+}. Further studies have also indicated that calcium has the same ameliorative effect for the acute toxicity of chloride in invertebrates (Mount et al., 1997; Soucek et al., 2011), although also not indicated by fixed calcium with titrated sodium. To a lesser extent, magnesium has also been described as producing an ameliorative effect on acute chloride toxicity. However, in the present study, the only cation binary mixture producing a less-than-additive effect was fixed calcium with increasing magnesium. These differences between acute toxicity and the chronic toxicity demonstrated in the present study may be a result of different mechanisms of action of these ions at varying combinations. These discrepancies between acute and chronic toxicity have been well documented for many contaminants. For example, copper is known to impair proper ionoregulatory capabilities at the gill following acute exposures; however, in chronic exposures, copper was shown to

accumulate within intestinal and liver tissues, creating lesions and nodules (Lauren and McDonald, 1985; McGeer et al., 2000; Bielmyer et al., 2005).

The lack of interpretability between acute and chronic studies suggests that chronic toxicity cannot be simply predicted or described by the acute toxicity of major ions. On the basis of environmentally relevant exposures, it is important to not discredit the effects that sub-lethal responses can have on populations of organisms. Furthermore, the majority of published literature characterizing the chronic toxicity of these major ions to invertebrate species focus on either single ion exposures (Lasier and Hardin, 2010; Elphick et al., 2011*a*; Elphick et al., 2011*b*). In aquatic systems, major ions do not occur as single constituents, but rather in mixtures. To better understand the effects that these major ions can have on the aquatic environment, the underlying mechanisms of the chronic effects of ion mixtures should be further examined.

Conclusions

The order of chronic toxicity for major ions (chloride, sulfate, bicarbonate, calcium, magnesium, and sodium) was $Ca^{2+} \geq Mg^{2+} \geq SO_4^{2-} > HCO_3^- > Cl^- > Na^+$, as described by their effects on *C. dubia* reproduction (EC_{50}). This order can further be extrapolated to indicate that divalent ions tended to be the most toxic on an ion-only basis, than anions. Results of binary mixture exposures indicated a variety of toxicity interactions including additive, greater-than-additive, and less-than-additive. Furthermore, these results differ from previously published literature describing the acute toxicity of these major ions, particularly for calcium, which is generally regarded as having an ameliorative effect on toxicity. These differences further emphasize the need

for additional information regarding chronic toxicity, and sub-lethal responses to major ions. Especially because many anthropogenic activities can potentially increase ion concentrations in freshwater systems above what is physiologically tolerable for these organisms. Furthermore, developing water quality criteria derived from chronic endpoints are essential for managing the release of ions both point (e.g. coal fired power plant effluents) and nonpoint (e.g. mountain top removal mining) sources.

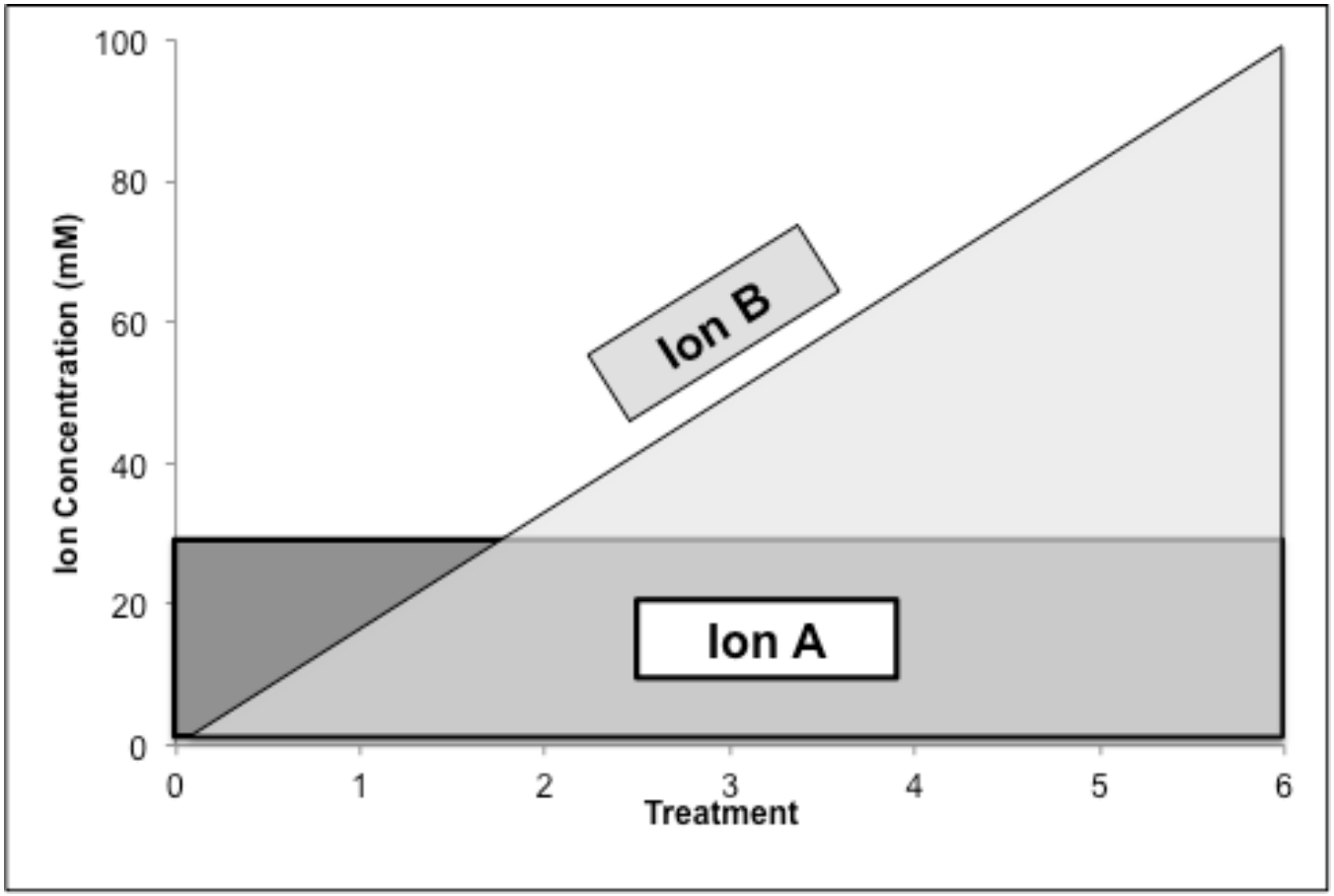

Figure 2.1. A graphical representation of the titration experimental design. One ion (Ion A) is held at a constant background concentration, a second ion (Ion B) increases in concentration across treatments.

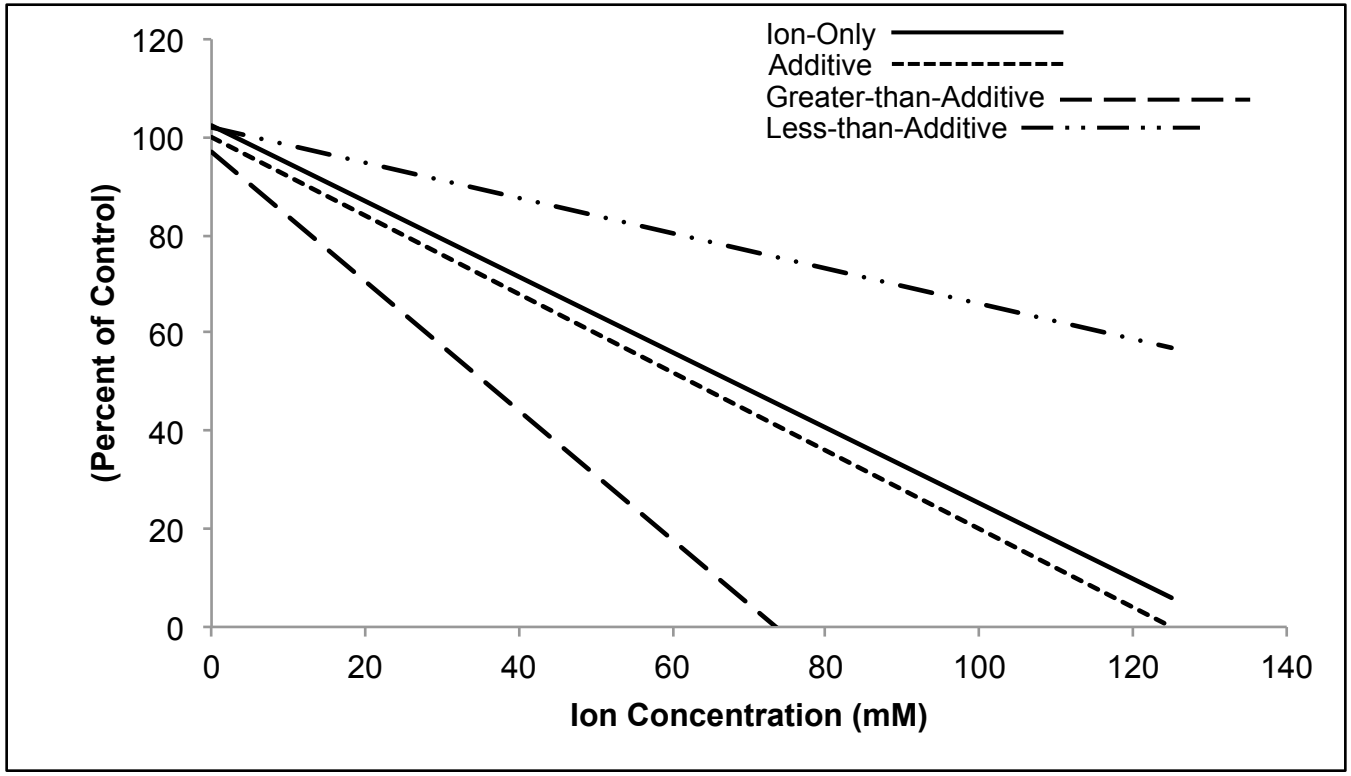

Figure 2.2. Slope analysis approach to determine contaminant mixture interactions. Results indicate additive, greater-than-additive, and less-than-additive.

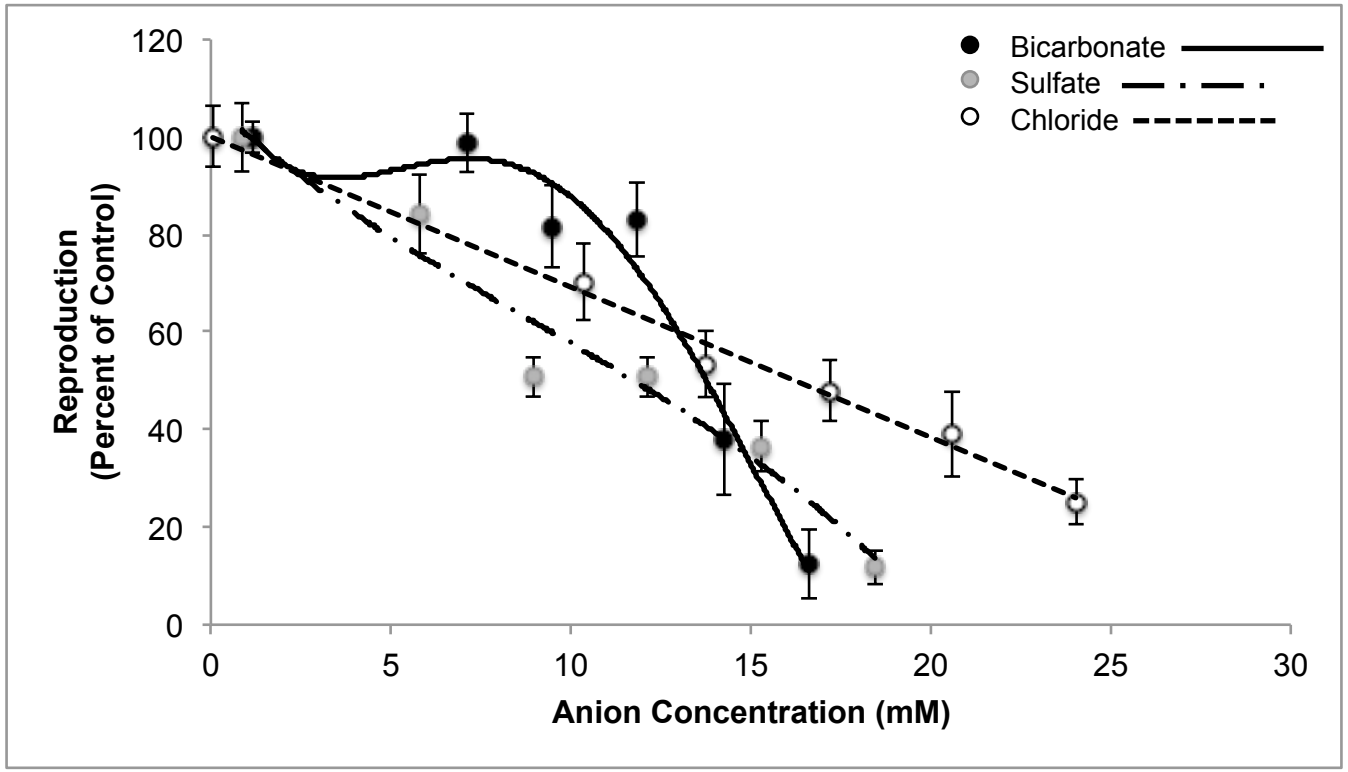

Figure 2.3. Average percent reproduction for *C. dubia* exposed to increasing concentrations of bicarbonate, sulfate, and chloride (as sodium salts). Individual data points represent the average reproduction for each treatment with error bars indicating 95% confidence intervals ($\alpha = 0.05$).

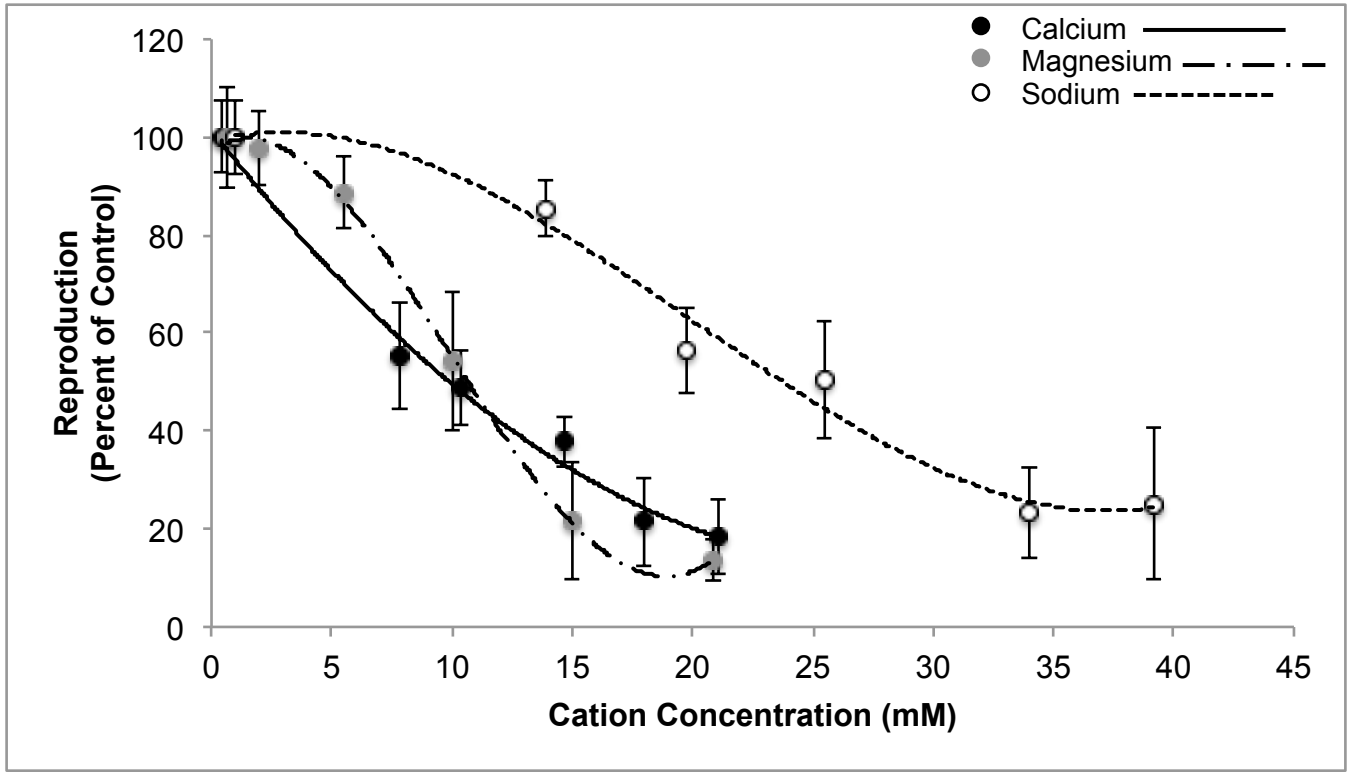

Figure 2.4. Average percent reproduction for *C. dubia* exposed to increasing concentrations of calcium, magnesium, and sodium (as chloride salts). Individual data points represent the average reproduction for each treatment with error bars indicating 95% confidence intervals ($\alpha = 0.05$).

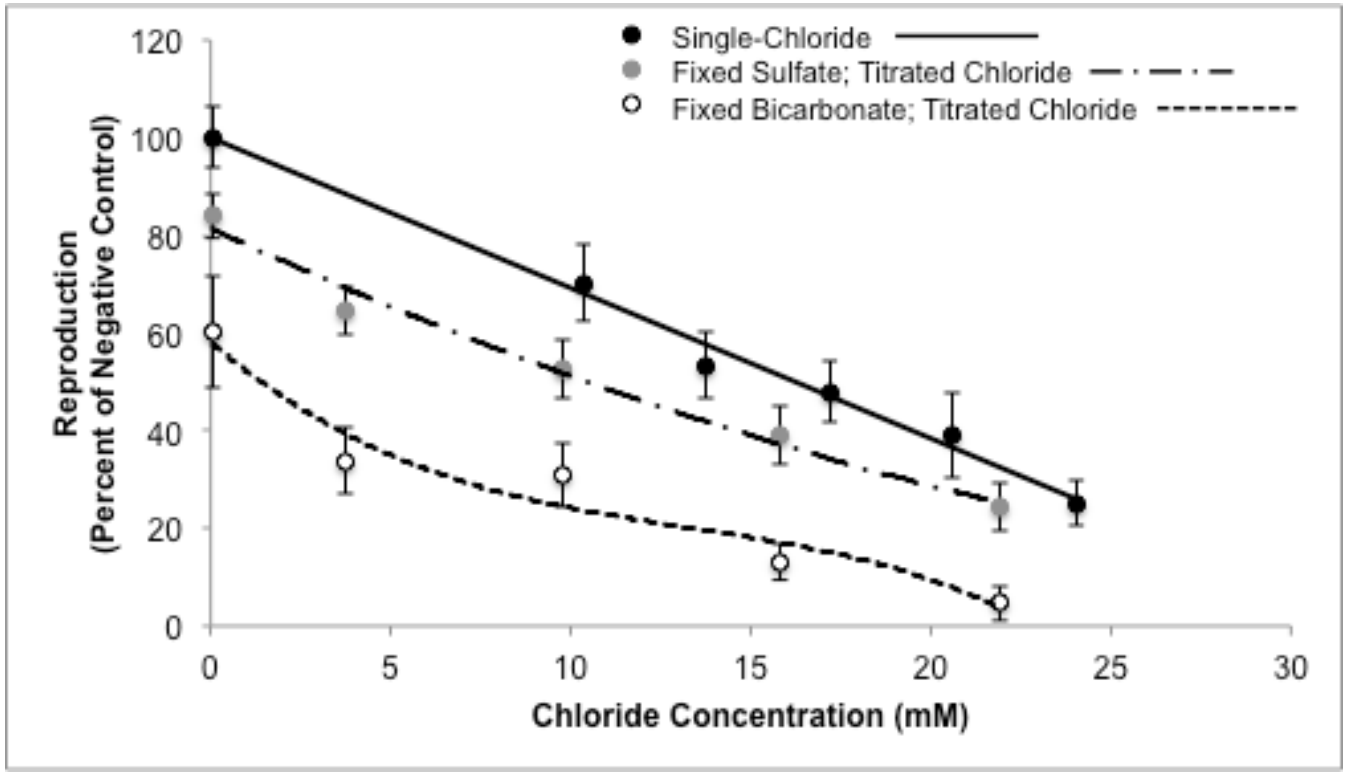

Figure 2.5. The effect of chloride with fixed sulfate (7.84 mM) and fixed bicarbonate (11.2 mM) on *C. dubia* reproduction. Data points represent average reproduction for each treatment, standardized to the negative control, with error bars indicating 95% confidence intervals ($\alpha = 0.05$).

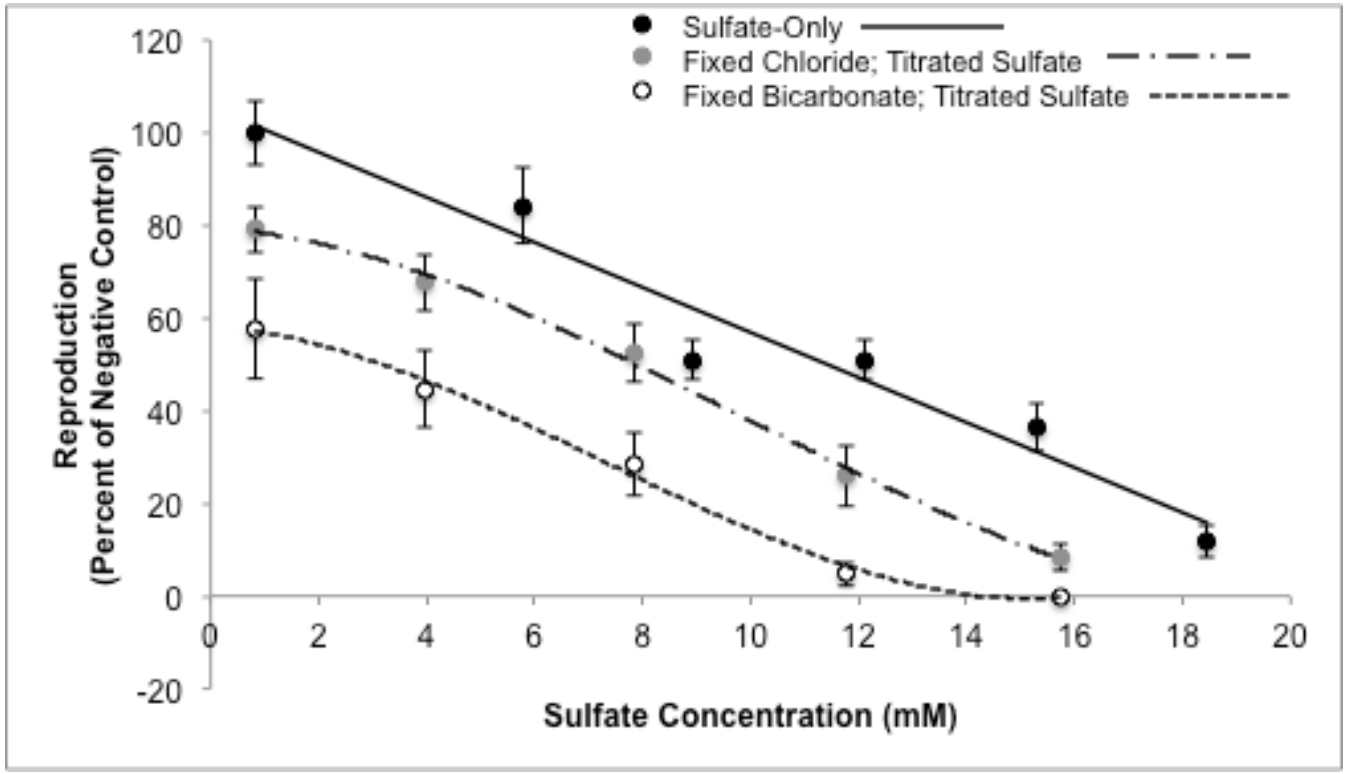

Figure 2.6. The effect of sulfate with fixed chlorde (9.81 mM) and fixed bicarbonate (11.2 mM) on *C. dubia* reproduction. Data points represent average reproduction for each treatment, standardized to the negative control, with error bars indicating 95% confidence intervals ($\alpha = 0.05$).

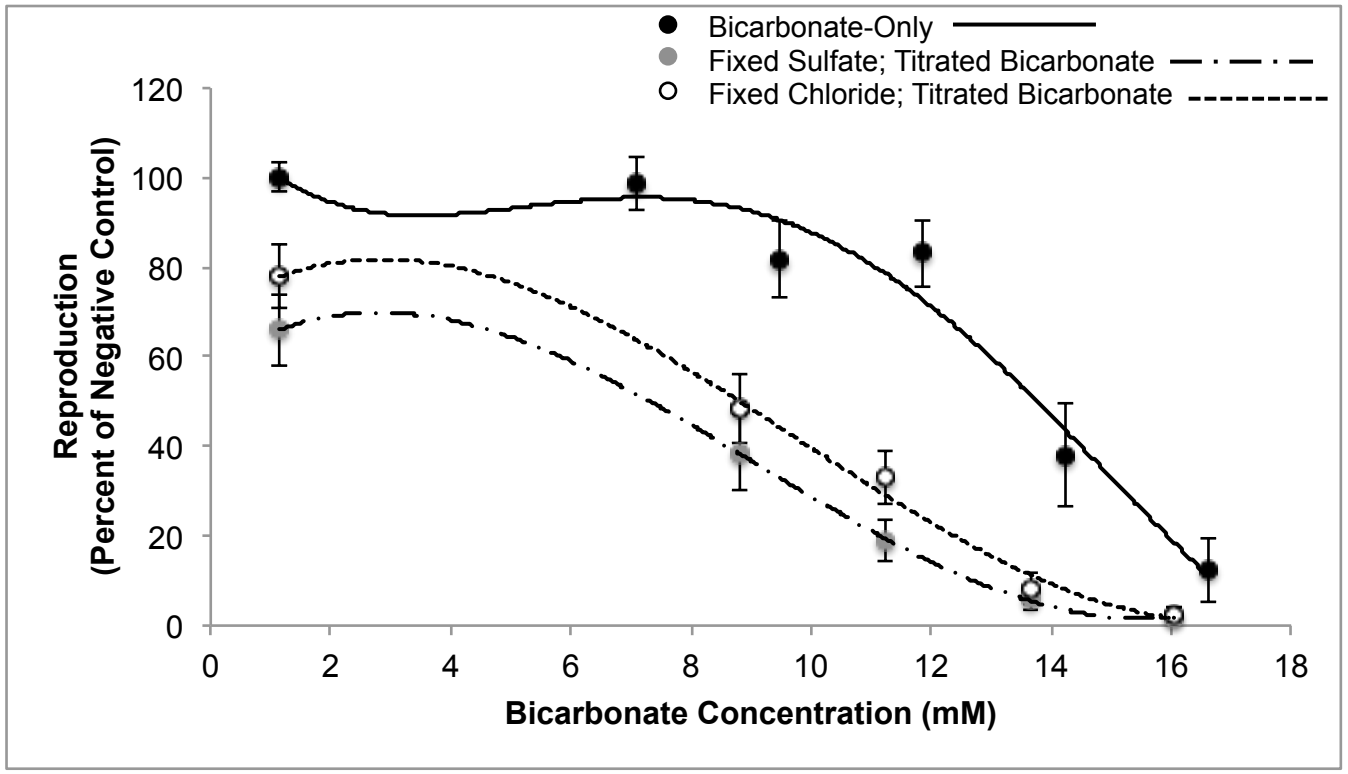

Figure 2.7. The effect of bicarbonate with fixed sulfate (7.84 mM) and fixed chloride (9.81 mM) on *C. dubia* reproduction. Data points represent average reproduction for each treatment, standardized to the negative control, with error bars indicating 95% confidence intervals ($\alpha = 0.05$).

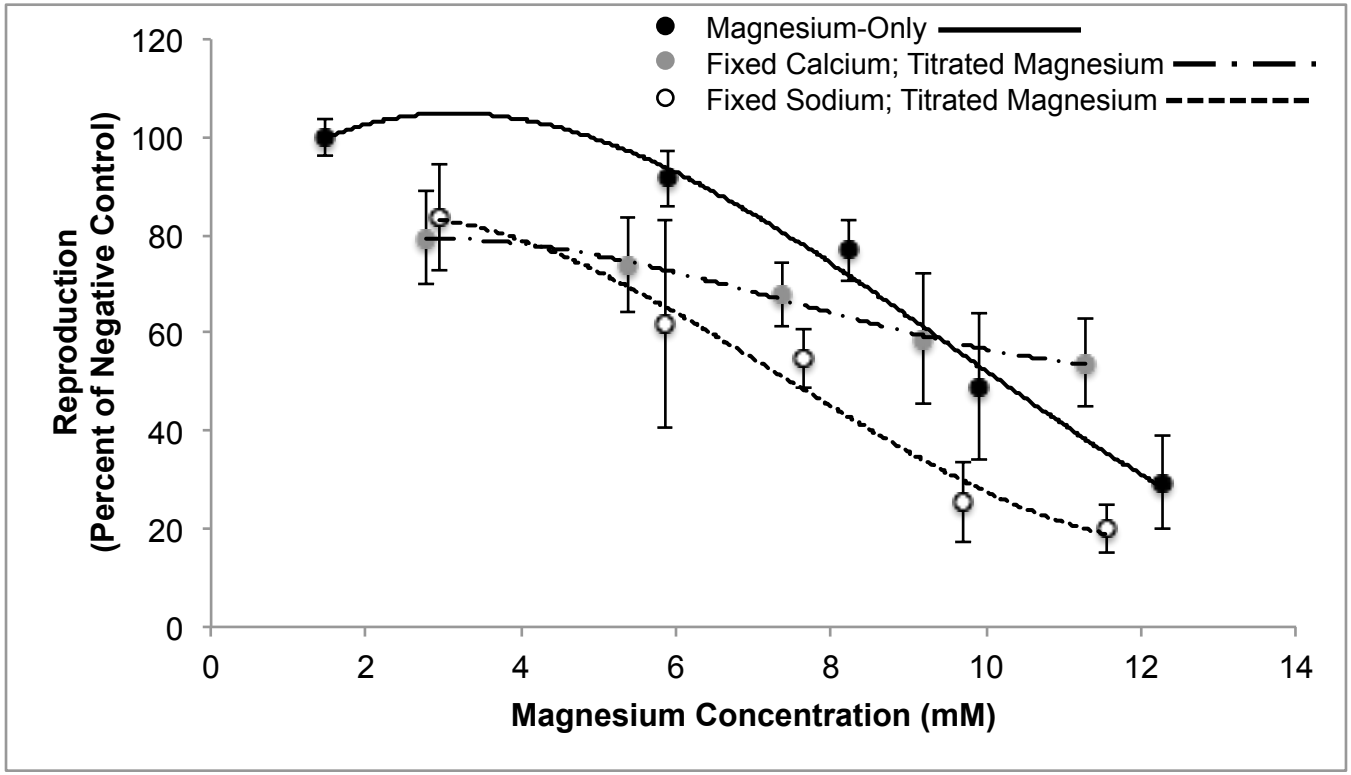

Figure 2.8. The effect of magnesium with fixed calcium (3.45 mM) and fixed sodium (30.8 mM) on *C. dubia* reproduction. Data points represent average reproduction for each treatment, standardized to the negative control, with error bars indicating 95% confidence intervals ($\alpha = 0.05$).

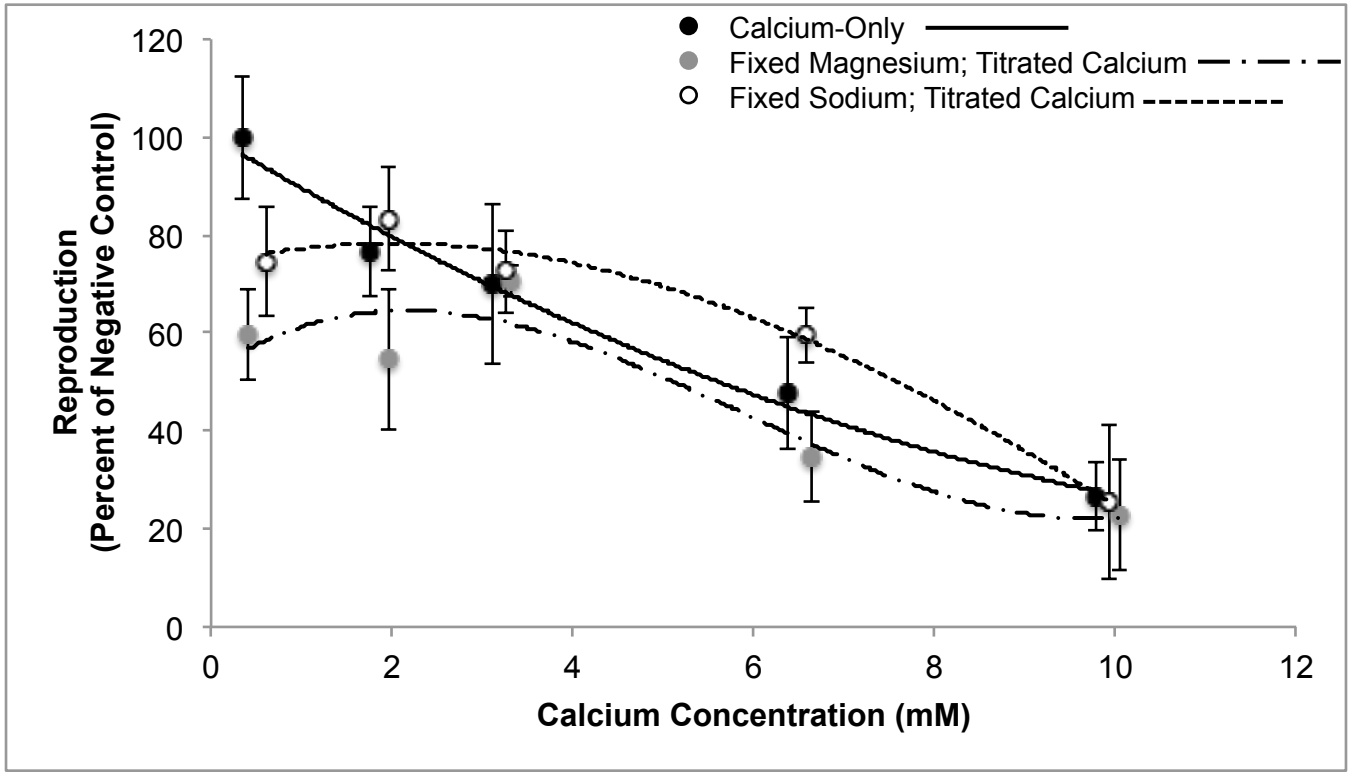

Figure 2.9. The effect of calcium with fixed magnesium (4.67 mM) and fixed sodium (13.2 mM) on *C. dubia* reproduction. Data points represent average reproduction for each treatment, standardized to the negative control, with error bars indicating 95% confidence intervals ($\alpha = 0.05$).

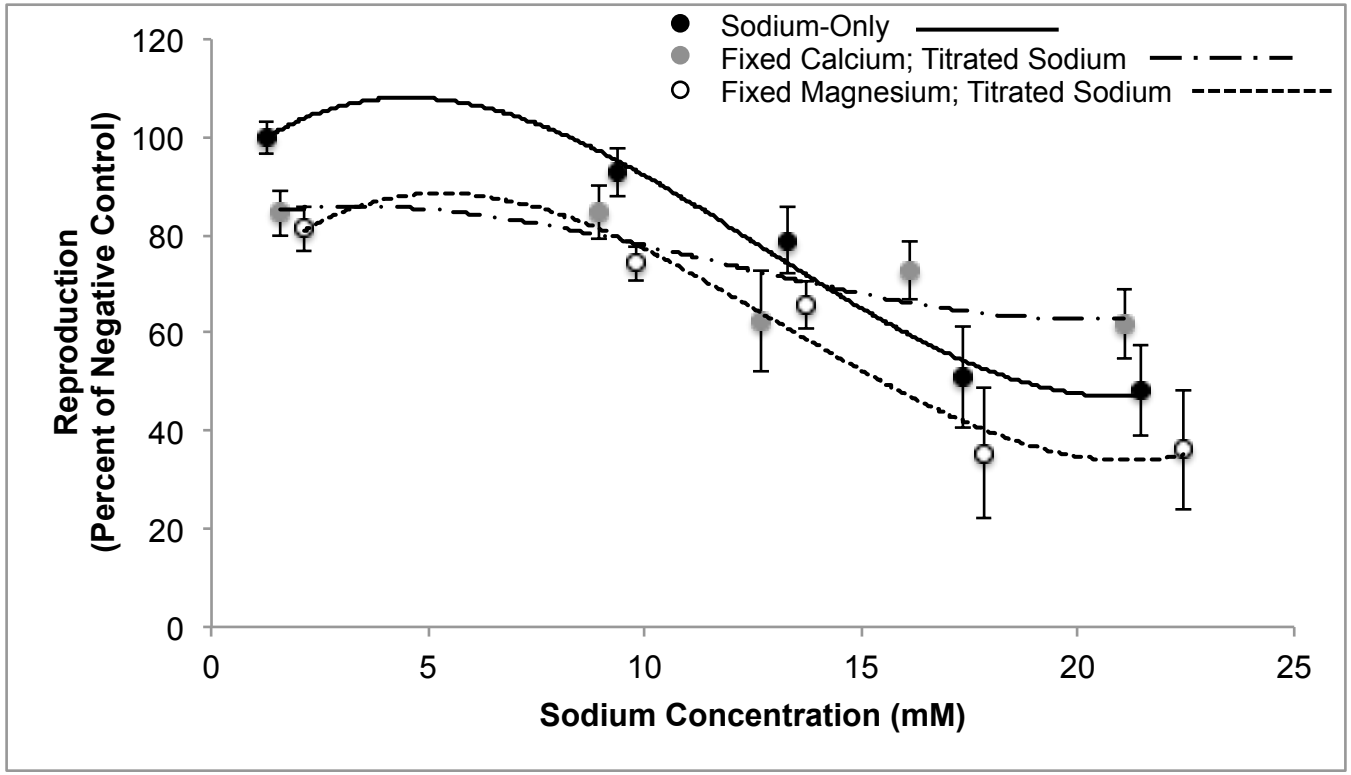

Figure 2.10. The effect of sodium with fixed calcium (3.26 mM) and fixed magnesium (4.27 mM) on *C. dubia* reproduction. Data points represent average reproduction for each treatment, standardized to the negative control, with error bars indicating 95% confidence intervals ($\alpha = 0.05$).

Table 2.1. EC_{50} values for *C. dubia* reproduction estimated from conductivity (µS/cm) and specific ion concentration (mM[a*].

Ion	EC_{50}	
	Conductivity (µS/cm)	Ion Concentration (mM)
Chloride	2122[c] (2028, 2217)	16.2[c] (15.4, 17.1)
Sulfate	2514[d] (2402, 2625)	12.0[a] (11.5, 12.6)
Bicarbonate	1322[a] (1287, 1357)	13.7[b] (13.3, 14.1)
Calcium	1649[b] (1500, 1799)	9.74[a] (8.54, 10.9)
Magnesium	1411[a] (1324, 1498)	10.4[a] (9.50, 11.5)
Sodium	2061[c] (1916, 2206)	23.6[d] (21.2, 26.1)

[a] Values in parenthesis indicate 95% confidence intervals for each EC_{50} value ($\alpha = 0.05$).

* Letters next to EC_{50}values for conductivity and ion concentration indicate statistical differences between single ions derived from a comparison between 95% confidence intervals.

Table 2.2. Statistical analysis of *C. dubia* anion binary mixture toxicity. Results include slope (mM), estimated EC_{50} value, R^2 for the best fitting line, *p* value calculated from anion-only and binary mixture comparisons, as well as the overall effect.

	Anion Binary Mixtures				
	Slope (mM)	**EC_{50}**	**R^2**	**P-Value**	**Effect**
Chloride-Only	-3.08 (-3.41, -2.75)	16.2 (15.4, 17.1)	0.739		
Fixed Sulfate; Titrated Chloride	-2.57 (-2.87, -2.25)	11.39 (10.4, 12.4)	0.580	0.0332*	Less-than-Additive
Fixed Bicarbonate; Titrated Chloride	-7.15 (-4.05, -1.45)	1.59 (0.145, 3.03)	0.439	0.0035*	Greater-than-Additive
Sulfate-Only	-4.94 (-5.30, -4.57)	12.0 (11.5, 12.6)	0.862		
Fixed Chloride; Titrated Sulfate	-6.75 (-9.08, -4.41)	8.0 (7.23, 8.78)	0.718	0.0568	Additive
Fixed Bicarbonate; Titrated Sulfate	-4.69 (-5.83, -3.55)	3.04 (1.40, 4.67)	0.465	0.7012	Additive
Bicarbonate-Only	-14.9 (-17.8, -11.9)	13.7 (13.3, 14.1)	0.746		
Fixed Chloride; Titrated Bicarbonate	-10.3 (-13.5, -7.14)	8.76 (7.95, 9.87)	0.639	0.0067*	Less-than-Additive
Fixed Sulfate; Titrated Bicarbonate	-8.05 (-12.2, -3.91)	7.32 (6.00, 8.64)	0.658	0.0103*	Less-than-Additive

Table 2.3. Statistical analysis of *C. dubia* cation binary mixture toxicity. Results include slope (mM), estimated EC_{50} value, R^2 for the best fitting line, *p* value calculated from anion-only and binary mixture comparisons, as well as the overall effect.

	Cation Binary Mixtures				
	Slope (mM)	**EC_{50}**	**R^2**	**P-Value**	**Effect**
Magnesium-Only	-10.2 (-12.3, -8.05)	9.92 (9.33, 10.5)	0.791		
Fixed Calcium; Titrated Magnesium	-3.97 (-7.69, -0.28)	★	0.283	0.0043*	Less-than-Additive
Fixed Sodium; Titrated Magnesium	-14.2 (-19.2, -9.25)	7.52 (6.57, 8.47)	0.601	0.5009	Additive
Calcium-Only	-6.36 (-10.0, -2.68)	5.59 (4.18, 7.00)	0.708		
Fixed Magnesium; Titrated Calcium	-10.7 (-13.7, -7.78)	6.5 (6.31, 6.69)	0.491	0.1251	Additive
Fixed Sodium; Titrated Calcium	-10.3 (-14.2, -6.32)	7.58 (5.54, 9.62)	0.532	0.2919	Additive
Sodium-Only	-6.91 (-9.96, -3.85)	18.3 (13.3, 15.3)	0.781		
Fixed Calcium; Titrated Sodium	-5.92 (-9.03, -2.82)	★	0.397	0.6603	Additive
Fixed Magnesium; Titrated Sodium	-7.45 (-10.9, -3.93)	14.9 (10.3, 19.7)	0.673	0.8204	Additive

★ The EC_{50} value for fixed calcium; titrated sodium and fixed calcium; titrated magnesium could not be estimated due to the lack of a 50% decrease in reproduction for both mixtures.

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CHAPTER THREE

THE CHRONIC TOXICITY OF MAJOR IONS AND ION BINARY MIXTURES TO *Pimephales promelas* (fathead minnow)

Introduction

Dissolved ions, commonly measured as conductivity (µS/cm) and Total Dissolved Solids (TDS), are natural constituents among aquatic systems. Freshwater systems typically exhibit very low conductivities and possess ionic compositions that are dependent on the surrounding geologic material of the water body. For example, a typical freshwater stream in the Central Appalachian Mountains is dominated by sulfate and bicarbonate with a conductivity of 62 µS/cm and a TDS concentration of 21 mg/L (Timpano et al., 2010). However, these characteristics are subject to change in areas impacted by anthropogenic activities such as agricultural irrigation, the application of salt to roads, mining operations, and coal-fired power plant effluents. Timpano et al. (2010) reported an increase in sulfate (SO_4^{2-}), calcium (Ca^{2+}) and potassium (K^+) concentrations of twenty-two mining sites in Virginia with a mean TDS concentration of 406 mg/L. Further studies describe similar trends, although to a higher degree with a mean TDS concentration of 1,165 mg/L and a substantial increase in sulfate concentration (Pond et al., 2008). Although conductivity and TDS are utilized in environmental settings in order to characterize changes from normal conditions, the use of these measurements to predict or describe potential toxic responses to elevated dissolved ions has been demonstrated to be a poor predictor of toxicity (Mount et al., 1997; Goodfellow et al., 2000; Kennedy et al., 2005; Mount et al., 2016). Inconsistencies between the toxicity of environmental

freshwater systems and conductivity have been observed. For example, the LC_{50} for *Ceriodaphnia dubia* (*C. dubia*) have been reported for coal mining effluent at 7,000 µS/cm (Kennedy et al., 2005), but only 3,000 µS/cm for irrigation drain waters (Dickerson et al., 1996). It is assumed that specific ion concentrations and ionic composition are better predictors of ion toxicity.

Current federal water quality criteria in the United States focus on chloride as a single ion (230 mg/L chronic; 860 mg/L acute); however, criteria for many other major ions including Na^+, HCO_3^-, SO_4^{2-} or even TDS currently do not exist. To develop water quality guidelines, it is important to consider not just acute toxicity, but more importantly chronic toxicity. The acute toxicity of ions and ions mixtures has been extensively investigated, with new reports being published frequently (Mount et al., 1997; Soucek and Kennedy, 2005; Soucek et al., 2011; Harper et al., 2014; Mount et al., 2016; Wang et al., 2016; Erickson et al., 2017; Erickson et al., 2018). Few studies have demonstrated the potential for chronic toxicity of ions, and more specifically, ion mixtures (Lasier and Hardin, 2010; Elphick et al., 2011*a*; Elphick et al., 2011*b*; Farag and Harper, 2014). Sub-lethal effects, however, should not be discounted. Unlike acute toxicity, such as mortality that can affect populations and ecosystems abruptly, chronic toxicity, including growth and reproductive effects, can disrupt ecosystem processes over time and may eventually lead to ecosystem collapse (Pond et al., 2008). The effects of chronic toxicity are often subtle, and can initially go undetected.

The relative order of acute toxicity for major ions has previously been reported as $K^+ > HCO_3^- \approx Mg^{2+} > Cl^- > SO_4^{2-}$ (Mount et al., 1997). It was further noted that Na^+ and

Ca^{2+} did not contribute to overall acute toxicity; however, current reports indicate that these cations may actually have a significant influence on ion mixture toxicity (Mount et al., 2016). Alternatively, the chronic toxicity of individual ions to *C. dubia* indicate an order of $Ca^{2+} \geq Mg^{2+} \geq SO_4^{2-} > HCO_3^- > Cl^- > Na^+$ (see Chapter Two). Differences exist between acute and chronic toxicity of ions and ion mixtures, and because most of the published literature emphasizes acute effects, further data is required in order to elucidate how these ions affect organisms on a sub-lethal basis. Additionally, little data has been generated regarding vertebrate effects (Farag and Harper, 2014; Wang et al., 2016). Therefore, the goal of this research was to characterize the chronic toxicity of major ions and ion binary mixtures to *Pimephales promelas* (fathead minnow), a vertebrate fish species. Results from this study will expand on the limited dataset regarding major ion effect on the growth of a higher-order species.

Materials and Methods

Pimephales promelas Maintenance and Culture

Larval *Pimephales promelas* (*P. promelas*) were obtained from an in-house culture of adult *P. promelas* at the Clemson Institute of Environmental Toxicology (CU-ENTOX, Pendleton, SC, USA). Adult fish were reared in a ~350-gallon freshwater recirculating system consisting of four troughs (retention time between 5-6 hours) with water quality parameters maintained as follows: Temperature: 25 ± 1°C; Alkalinity: 56 mg/L as $CaCO_3$; Hardness: 100 mg/L as $CaCO_3$; pH: 7.5. Reproduction was monitored daily by the presence of eggs on polyvinyl chloride tiles, which were placed in each trough to enhance reproduction. Tiles with eggs were immediately removed from the

recirculating system and placed in a separate holding tank containing U.S. EPA reconstituted moderately hard water (18 MΩ•cm ultrapure water, 96 mg/L $NaHCO_3$, 60 mg/L $CaSO_4 \bullet 2H_2O$, 60 mg/L $MgSO_4$, and 4.0 mg/L KCl) and an air stone to redistribute any static water (U.S. EPA, 2002). Eggs were gently rinsed twice daily with deionized water, any non-viable eggs were discarded, and the number of hatched fry was recorded.

Test Solutions

Stock solutions of each ion were made daily with ACS certified salts and reconstituted moderately hard water. Prepared stock solutions included sodium chloride (CAS 7647-14-5), calcium chloride (CAS 10035-04-8), magnesium chloride (CAS 7791-18-6), sodium bicarbonate (CAS 144-55-8), and sodium sulfate (CAS 7757-82-6) (purchased from Fisher Scientific, Atlanta, GA, USA). The appropriate amount of each stock solution was added to 2-L of reconstituted moderately hard water to create test solutions. After preparation, solutions were allowed to come to equilibrium for 24-hours prior to use and maintained at a temperature of 25 ± 1 °C. Test solutions were created daily. A small sample was collected for each test solution to quantify accurate ion concentrations using ICP-MS.

Bioassay Procedure and Experimental Design

Procedures for conducting 7-day *P. promelas* bioassays followed the U.S. EPA short-term chronic toxicity estimation guidelines framework (U.S. EPA, 2002). Specifically, each replicate contained ten < 24-hour old larval *P. promelas*, with five replicates per treatment. During test initiation, the ten fish were transferred at random to one 600-mL polyethylene beaker containing 300 mL test solution. Test chambers were

kept in a temperature (25 ± 1°C) and light (16:8 light/dark cycle) controlled room with water renewals occurring every 24-hours. During renewals, water quality measurements were recorded for both the initial test solution (prior to organism introduction) and final test solution (24-hours after exposure). These measurements included temperature (°C), dissolved oxygen (mg/L), pH, alkalinity (mg/L as $CaCO_3$), hardness (mg/L as $CaCO_3$), total ammonia (mg/L), and conductivity (µS/cm). Any deceased test organisms were promptly removed from the test chamber.

Test organisms were fed twice daily, at 4-hour intervals, a concentrated solution of <24-hour old *Artemia* nauplii cultured as described by the U.S. EPA (2002). On day seven of the bioassay, fish were euthanized using a 1.0 g/L MS-222 solution buffered with $NaHCO_3$ to a pH of 7.5. Each replicate was filtered using a 2-inch borosilicate cylinder affixed with 120 µm mesh and rinsed with approximately 50 mL deionized water. Organisms were then transferred to separate pre-weighed aluminum tins for each replicate and placed in a drying oven at 60°C for 24-hours. Average fish dry weights were calculated by subtracting the initial tin-only weight from the final dry weight of the tin + fish, and then dividing by the number of fish placed in each tin (the number of fish that survived per replicate).

Single-ion bioassays were performed initially to determine the appropriate concentration of each ion needed for binary mixtures. Following single-ion bioassays, binary mixture bioassays were conducted. For these mixture exposures, a titration experimental design was employed. This type of design consists of one ion being held at a constant background concentration (Ion A), while the second ion (Ion B) increases in

concentration across treatments 3.1). Each bioassay included a negative control treatment that only contained reconstituted moderately hard water. Results of bioassays were accepted if survival was ≥80% in the negative control. A second treatment, identified as the positive control, was composed of reconstituted moderately hard water and the fixed concentration of Ion A. Subsequent treatments still contained the reconstituted moderately hard water, fixed concentration of Ion A, and increasing concentrations of Ion B. One single-ion bioassay was performed in conjunction with one binary mixture bioassay. This means that at any given time, two to four bioassays were being conducted simultaneously. The concentration of the titrated ion (Ion B) was the same for both single-ion and binary mixture bioassays.

Chemical Analysis

Small aliquots, filtered through a 0.45 μm filter, were collected daily for each control and treatment in order to confirm ion concentrations. Samples were placed in a 15 mL plastic tube and then diluted within the detection limit to a pH <3.0 using HNO_3 and ultrapure water (18 MΩ resistivity). Cation concentrations were then confirmed using ICP-MS at the Clemson University Institute of Environmental Toxicology. Measured cation concentrations were used to calculate anion concentrations. For example, chloride concentrations were calculated based on the confirmed concentration of sodium, but corrected for the chloride present in the reconstituted moderately hard water.

Statistical Analysis

For all statistical analyses, JMP® 11.0.0 was utilized. All results, as weight (mg), were standardized to the average weight (mg) of the negative control treatment (the treatment only containing moderately hard water) for each bioassay. In doing so, organismal randomization and health were accounted for. Each concentration-response curve was analyzed by both linear regressions and Hill equations (Logistic 4P). The best-fit curve, determined by the fit with the highest R^2 values, was used in all subsequent data analysis.

Estimations of Effective Concentrations for a 50% decrease in reproduction (EC_{50}), and the associated 95% confidence intervals ($\alpha = 0.05$), were calculated by inverse interpolation. Conductivity (μS/cm) and specific ion concentration (mM) for ion-only bioassays were used in estimations of EC_{50} values. Non-overlapping 95% confidence intervals indicated significant differences between ion-only EC_{50} values.

Depending on the best-fit curve, either overall linear slopes or approximate Hill slopes were compared using Analysis of Covariance (ANCOVA) to determine significant differences between ion-only and ion binary mixture concentration-response curves. For graphing purposes, polynomial fits are represented for Hill equations. However, slopes and EC_{50} values were estimated from Hill equations. Analyses indicating no statistical differences resulted in additive interactions for the binary mixture. In contrast, statistical differences could either indicate less-than-additive interactions where the binary mixture produces a shallower slope than the ion-only slope, or greater-than-additive where the binary mixture produces a steeper slope than the ion-only slope (Figure 3.2).

Results

Ion-Only Toxicity

Conductivity (μS/cm) and specific ion concentration (mM) were used to estimate Effective Concentrations (EC_{50}) that reduced *P. promelas* growth by 50% compared to control growth. Effective concentrations estimated from conductivity indicated a relative order of toxicity as $HCO_3^- > SO_4^{2-} \geq Mg^{2+} > Na^+ \geq Cl^- > Ca^{2+}$. Significant differences occurred between conductivity EC_{50} values, as determined by overlapping 95% confidence intervals (Table 3.1). For example, calcium was significantly less toxic than any other ion. Conversely, estimations derived from specific ion concentrations indicated an order of $SO_4^{2-} \geq Mg^{2+} > HCO_3^- \geq Ca^{2+} > Cl^- > Na^+$. Significant differences due to non-overlapping confidence intervals were also noted for these values (Table 3.1). Due to a large degree of mortality in most exposures, 7-day Lethal Concentrations, resulting in 50% decrease in survival (LC_{50}), were also calculated (Table 3.1).

Sulfate (Figure 3.3; slope: –9.08; R^2: 0.859) had the largest concentration-response slope suggesting that it has the greatest effect on *P. promelas* growth per millimolar basis compared to both bicarbonate (slope: –1.54; R^2: 0.939; *p* value: 0.0006*) and chloride (slope: –3.33; R^2: 0.856; *p* value: 0.0188*). Furthermore, the chloride concentration-response slope was significantly different from the slope of bocarbonate (*p* value: 0.002*). Bicarbonate had the shallowest slope of the three anions (Figure 3.3). Similar to growth effects, sulfate also resulted in the largest decrease in survival (slope: –4.76; R^2: 0.799) and was significantly different from bicarbonate (slope: –1.26; R^2: 0.936; *p* value: 0.0147*) and chloride (slope: –1.28; 0.725; R^2: 0.725;

p value: 0.0296*). Chloride and bicarbonate had similar concentration-response slopes (*p* value: 0.965); however, LC_{50} values were significantly different (Figure 3.4).

Slopes calculated for cation-only concentration-response curves indicated that sodium (slope: –7.12; R^2: 0.728) had the least overall effect on *P. promelas* growth compared to magnesium (Figure 3.5; slope: –10.2; R^2: 0.861; *p* value: 0.0005*). The concentration-response slope measured for sodium was not significantly different from calcium (slope: –9.01; R^2: 0.653; *p* value: 0.364), nor was calcium significantly different from magnesium (*p* value: 0.1136) (Figure 3.5). Although the effect on *P. promelas* growth were similar of sodium, calcium and magnesium on *P. promelas* growth were similar on a milli-molar basis, EC50 values were significantly different. Effects on survival of *P. promelas*, for sodium, calcium, and magnesium were similar to those on growth (Figure 3.6).

Overall slopes for both cations and anions suggest that divalent ions, including calcium, sulfate, and magnesium, resulted in the largest effect on both *P.* promelas survival and growth. However, these results were not similar to the relative order of toxicity developed from EC_{50} or LC_{50} values. This indicates that the potency of these ions does not correspond with the effective or lethal concentrations.

Anion Binary Mixture Toxicity

The concentration-response curve for bicarbonate in the presence of 14.7mM sulfate resulted in an additive interaction for effects in both growth (slope: –1.81; R^2: 0.903; *p* value: 0.099) and survival (slope: –1.78; R^2: 0.918; *p* value: 0.613) of *P. promelas*. Similarly, the addition of 35mM chloride to titrated bicarbonate also resulted

in an additive interaction (growth - slope: –1.53; R^2: 0.977; *p* value: 0.123; survival - slope: –1.44; R^2: 0.926; *p* value: 0.772) (Figure 3.7; Figure 3.10; Table 3.2). Regarding growth (*p* value: 0.104) and survival (*p* value: 0.177), there was no significant difference between fixed chloride with titrated bicarbonate, and fixed sulfate with titrated bicarbonate, suggesting that the addition of either anion to bicarbonate results in a similar response. Similar growth effects resulted from exposures with titrated sulfate in the presence of both 25.2mM chloride (slope: –3.12; R^2: 0.876; *p* value: 0.927), and 22.7mM bicarbonate (slope: –3.51; R^2: 0.873; *p* value: 0.511) (Figure 3.9). Moreover, survival data indicate additive interactions for these mixtures as well (Figure 3.12; Table 3.4). Although the two sulfate-only survival concentration-response curves produced resulted in statistically different slopes (*p* value: 0.0329*; * indicating a significant difference), the results were not different between the two mixtures.

Additive growth effects were also a result of titrated chloride in the presence of 22.7mM bicarbonate (slope: –1.98; R^2: 0.924; *p* value: 0.751). This additive interaction did not extend to the survival effects of the same mixture. Instead, survival was significantly more for the mixture of fixed bicarbonate with titrated chloride (slope: –0.988; R^2: 0.935; *p* value: 0.0178*), than for chloride-only (slope: –2.10; R^2: 0.861). Less-than-additive interactions were also concluded for both growth (slope: –2.23; R^2: 0.959; *p* value: 0.0178*) and survival (slope: –1.05; R^2: 0.946; *p* value: 0.0497*) effects for chloride in the presence of 13.9mM sulfate (Figure 3.8; Figure 3.11; Table 3.3).

Cation Binary Mixture Toxicity

Mixtures containing titrated calcium in the presence of 18.4mM magnesium resulted in statistically similar slopes (slope: –2.49; R^2: 0.564; *p* value: 0.921) for growth effects when compared to calcium-only (slope: –2.49; R^2: 0.926), indicating additivity (Figure 3.13). Although not as strong, a comparison of the survival concentration-response curves for the same mixture also indicated additive effects (*p* value: 0.086). This slight similarity for survival effects could indicate that a shift towards greater-than-additive, or an increase in toxicity due to this cation mixture (Table 3.5). Survival data reveal that as calcium exceeds 28.9mM, in the presence of 18.4mM magnesium, a considerable reduction from 86% to 12% in survival occurs (Figure 3.16), although not significant. Further additive interactions resulted from titrated chloride in the presence of 43.7mM sodium for both growth (*p* value: 0.690) and survival (0.521) effects (Table 3.5).

The concentration-response curve for increasing sodium concentrations in the presence of 28.7mM calcium (slope: –1.16; R^2: 0.745), indicated additive interactions for both growth (*p* value: 0.211) and survival (*p* value: 0.529) effects (Table 3.7; Figure 3.15; Figure 3.18). Combinations of sodium in the presence of 16.4mM magnesium further demonstrated additive responses (Table 3.7).

A less-than-additive interaction between titrated magnesium in the presence of 29.2mM calcium was demonstrated for both growth (slope: –0.619; R^2: 0.113; *p* value: 0.009*) and survival (slope: –3.78; R^2: 0.876; *p* value: 0.0363*) effects. Both responses suggest that calcium decreases magnesium toxicity, and has this same effect for survival and growth. Although the addition of 40.0mM sodium to titrated magnesium produced

the same protective effect as calcium for survival, and resulted in less-than-additive interactions (Figure: 3.17; slope: –3.74; R^2: 0.877; *p* value: 0.0005*), this effect did not extend to growth effects (slope: –3.10; R^2: 0.149; *p* value: 0.315) (Table 3.6; Figure 3.14). The statistically similar slopes indicate additive effects on growth for magnesium in the presence of sodium.

Discussion

Conductivity, a measurement that combines all ions into one parameter, has been previously used as a surrogate to explain major ion toxicity (Kennedy et al., 2005; Pond et al., 2008; U.S. EPA, 2011). By describing ion toxicity on the basis of conductivity alone assumes that all ions have a similar chemical activity and effect on biological systems. As such, it would be expected that there would be no significant differences between growth effects, described by their EC_{50} values estimated from conductivity. Conversely, utilizing specific ion concentration as a means to explain toxicity would assume that each ion has the potential to exert its own specific effect at the site of action, most prominently the gill. In this case, there should be a clear toxicity gradient in which some ions produce a more considerable response than others. Results from the present study indicate a significant difference between ions, and their corresponding EC_{50} values that were estimated from conductivity. Although some ions demonstrated similar responses, it is important to note that these are values estimated from single-ion exposures. In cases where multiple ions exist at elevated concentrations, their electrochemical behaviors and interactions can change, ultimately influencing the solution conductivity. These results are comparable to previous studies that have

suggested conductivity is not the best approach for estimating ion toxicity (Mount et al., 1997; Kennedy et al., 2003; Timpano et al., 2010; Soucek et al., 2011; Kunz et al., 2013; Mount et al., 2016).

Initial ion-only bioassays identified sulfate, magnesium and bicarbonate as being the most toxic of the six ions tested, as described by their EC_{50} values. Calcium demonstrated a similar toxicity as bicarbonate, indicating that a 50% decrease in growth occurred at comparable ion concentrations. Both sodium and chloride were identified as the least toxic for both survival and growth of fathead minnows. Direct comparisons between the estimated EC_{50} values in the present study, and those described in the published data could not be made. Farag and Harper (2014) estimated a *P. promelas* IC_{20} for bicarbonate as 8.17mM (95% confidence intervals, 7.65 – 8.70mM bicarbonate). This estimate is roughly 42% lower an EC_{20} value estimated for the present study: 14.1mM HCO3- (95% confidence intervals, 11.4 – 16.9mM bicarbonate). A seven-day LC_{50} of 22.1mM bicarbonate (17.9 – 25.1mM bicarbonate) was also reported, and was approximately 47% lower than the present study (Table 3.1). Furthermore, other studies have reported lower seven-day LC_{50}s for *P. promelas* as 5.28 – 6.71mM sulfate (Wang et al., 2016). Typical 96-hour acute toxicity exposures of NaCl, Na2SO4, and NaHCO3 as single salts to *P. promelas* resulted in 109mM chloride (103.1 – 121.1mM chloride), 56mM sulfate (47.8 – 70.4mM sulfate) and 10.5mM bicarbonate (3.69 – 14.5mM bicarbonate) as LC_{50} values respectively (Mount et al., 1997). These differences between anion-only exposures could be explained by differences in testing procedures and the age of the fish at test initiation. For example, Mount et al. (1997) initiated testing with larval

fish between one and seven days post-hatch, while Farag and Harper (2014) initiated testing at < 48-hours post-hatch. Additionally, Mount et al. (1997) utilized fish that had been cultured in-house, while Farag and Harper (2014) had to transport fish to the testing location. The age of fish utilized in exposures may be of greater importance when determining effects due to increased salinity. Previous studies have indicated that some fish, particularly tilapia and killifish, do not possess functional gills until approximately 96-hours post-hatch (Ayson et al., 1994; Katoh et al., 2000). The ability for the yolk sac in *P. promelas* to regulate ion intake at the same rate and capacity as a fully functioning gill is unknown. However, this could be a reason for differences between anion results in these studies. Cation-only seven-day LC_{50} values estimated in the current study were fairly comparable to the five-day LC_{50} values reported by Mount et al. (1997), suggesting that *P. promelas* may regulate cations to a greater extent than anions.

It is also important to note that the LC_{50} values were not significantly different from EC_{50} values estimated for sulfate, bicarbonate, magnesium, and calcium. This may suggest that for these ions in particular, the mechanism of action resulting in acute toxicity may be the same for chronic, specifically growth. These ions play many important physiological roles in freshwater organisms, one of which is the formation of electrochemical gradients within cells. By generating these gradients, they ultimately produce a delicate balance that controls water and ion movement within the organism. Because the internal ion concentration of freshwater organisms is typically greater than their external environment, it is necessary for them to utilize active transport through a system of pumps and transporters within the gills to translocate ions (Ahearn et al., 1998;

Perry et al., 2003). It is possible that these organisms increase the energy expenditure used to properly ionoregulate during periods of elevated salinity, while decreasing the amount of energy used for reproduction and growth. Once the ionoregulatory capabilities of the organism have been exhausted, the overall function of the gill may cease (Greenaway P, 1979). Currently, the precise mechanism behind the sub-lethal effects of these ions is unknown. Determining that mechanism may help identify the way in which these ions interact and contribute to the understanding and prediction of their toxicity in environmental situations.

Binary mixture toxicity for major anions and cations was identified through a titration experimental design. The titration experimental design is useful in identifying how the toxicity of one ion can be influenced by the presence of a second ion. In the present study, most ion binary mixtures resulted in an additive response, meaning that the addition of a second ion did not alter the toxicity of the first. Previous studies have suggested that contaminants with similar concentration-response curves, resulting in additive interactions, may have similar modes of action (van der Geest et al., 2000). This could further exemplify that these ions create a high-stress environment for freshwater organisms, where more energy is utilized in order to ionoregulate properly regardless of the ions present.

However, growth effects showed a shift from additivity when sulfate was introduced with chloride (fixed sulfate with titrated chloride). Similar results have been demonstrated in invertebrate species. More specifically, a decrease in toxicity occurred in *Hyallela azteca* (*H. azteca*) after exposure to chloride (5 – 25 mg/L chloride) with the

addition of fixed sulfate (Soucek, 2007). However, this same alleviation in toxicity did not occur in embryonic *P. promelas* when exposed to fixed sulfate with increasing concentrations of chloride (10 – 25 mg/L chloride) over fourteen-days (Wang et al., 2016). In the fourteen-day exposure, the chloride concentrations utilized were much lower than the current study. In fact, chloride concentrations were only increased from 0.14 to 0.71mM, whereas, in the present study, chloride was between 0.05 and 3.10mM. For this particular mixture, sodium is added at a 1:2 molar ratio due to sodium chloride and sodium sulfate. An increase in the external concentrations of both chloride and sodium has been shown to increase the passive loss of both ions (Bourguet et al., 1964). A passive loss means that the energy required to remove excess sodium and chloride from the plasma of freshwater fishes decreases, possibly meaning that more energy is available for other functions. So quite possibly, the alleviation in toxicity of sulfate and chloride is not related to them directly, but more so the high concentration of sodium. However, this same reduced toxicity did not occur in mixtures containing sodium bicarbonate and sodium sulfate, which had similar sodium concentrations. So another explanation may lie in sulfate directly. Although the exact mechanism of sulfate toxicity is mostly unknown, it may block the ability of chloride to gain access to the gill due to its large molecular size, leading to an overall decrease in chloride toxicity. This may explain why the same result did not occur for sulfate mixed with bicarbonate. Bicarbonate is excreted through a chloride/bicarbonate exchanger located on the apical membrane of the gill after CO_2 waste is converted into H^+ and HCO_3^- by the carbonic anhydrase enzyme to control acid-base balance within the organism. However, in this particular mixture,

bicarbonate increases while chloride remains low. The ability for the chloride/bicarbonate exchanger to remove bicarbonate from the chloride cell may be reduced from a lack of chloride, as well as the high bicarbonate concentration.

Calcium also seemed to provide a protective effect when introduced to magnesium (fixed calcium with titrated magnesium). The protective effect of calcium has been previously described in algae, aquatic plants, a snail species (*Amerianna cumingi*), the Northern trout gudgeon (*Morurnda mogurnda*) and *C. dubia* (Mount et al., 1997; van Dam et al., 2010, Mount et al., 2016; Wang et al., 2016). Magnesium, although an important ion in many cellular processes such as activation of enzymes and protein synthesis, is also relatively toxic at low concentrations (Jahnen-Dechent and Ketteler, 2012). As such, the control of magnesium within the organism is especially controlled (Cowey et al., 1997). In fact, it has been reported in stickleback (*Gasterosteus aculeatus*), Mozambique Tilapia (*Oreochromis mossambicus*) and goldfish (*Carassius auratus*) that as external magnesium concentrations increase, plasma magnesium remains relatively constant (Wendelaar Bonga, 1978; Wendelaar Bonga et al., 1983; Olivereau et al., 1987). One of the key ways magnesium exerts its toxicity is by blocking the uptake of calcium, eventually producing a calcium deficiency (Hardwick et al., 1991). However, as the concentration of calcium increases, it may compete with magnesium at binding sites located on the chloride cells of fish, ultimately reducing magnesium uptake and toxicity (van Dam et al., 2010). The fact that sodium does not compete for the same binding sites as calcium and magnesium may explain why calcium did not have the same ameliorative effect on sodium as it did for magnesium.

Attention has been directed towards the toxicity of major ions primarily due to an increase in these constituents from coal-mining, coal-fired power generation, agricultural irrigation, as well as many other anthropogenic activities. Due to the numerous anthropogenic sources that contribute to increasing ions, it has become critical to create watershed management practices in order to maintain a healthy aquatic ecosystem. While the mechanisms behind their toxicity is, for the most part, unknown, considerable efforts have given rise to significant data illustrating their effects on survival. Limited work has been conducted to better understand sub-lethal effects, such as growth and reproduction. However, inconsistencies between previously reported data, and those presented in the present study indicate the complexities that are associated with these ion mixtures. Variations between concentration-response curves may demonstrate a difference in the mechanisms that produce sub-lethal effects. The development of a predictive model for environmental monitoring purposes, similar to the BLM model for metals toxicity, would help reduce the need for animal testing, as well as site-specific water quality criteria. Understanding some of the basic mechanisms behind these toxic effects is fundamental in creating accurate predictive models.

Conclusions

Significant differences between the toxicity of major ions, described by conductivity, further emphasize that conductivity may not be the most accurate predictor of ion toxicity. As such, the remaining analysis focused on specific ion concentrations (mM). The chronic toxicity of major ions to *Pimephales promelas* indicated an order of toxicity as $SO_4^{2-} \geq Mg^{2+} > HCO_3^- \geq Ca^{2+} > Cl^- > Na^+$. The most toxic of the six ions tested

included divalent ions, with bicarbonate not being significantly different from calcium. Binary mixture exposures concluded in mostly additive interactions between the six ions; however, fixed sulfate with increasing concentrations of chloride, as well as fixed calcium with increasing concentrations of magnesium resulted in less-than-additive interactions. These results demonstrate the complex nature of ion toxicity, especially sub-lethal effects, and the potential difficulty with developing predictive models. Incorporating the basic mechanisms of sub-lethal responses to ions and ion mixtures will significantly increase the accuracy and validity of these models, as well as decrease the need for site-specific water quality criteria.

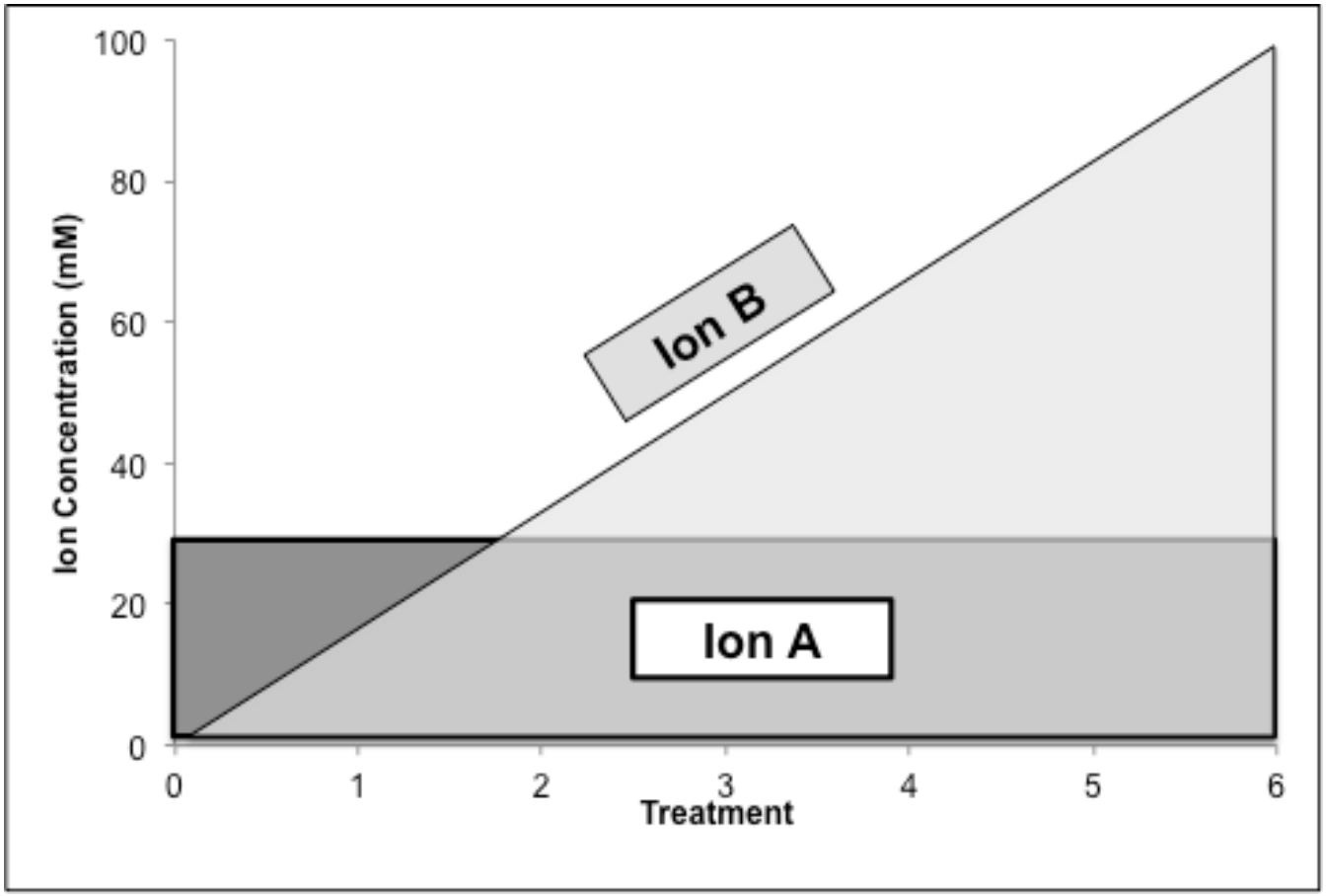

Figure 3.1. **A graphical representation of the titration experimental design**. Where one ion (Ion A) is held at a constant background concentration, a second ion (Ion B) increases in concentration across treatments.

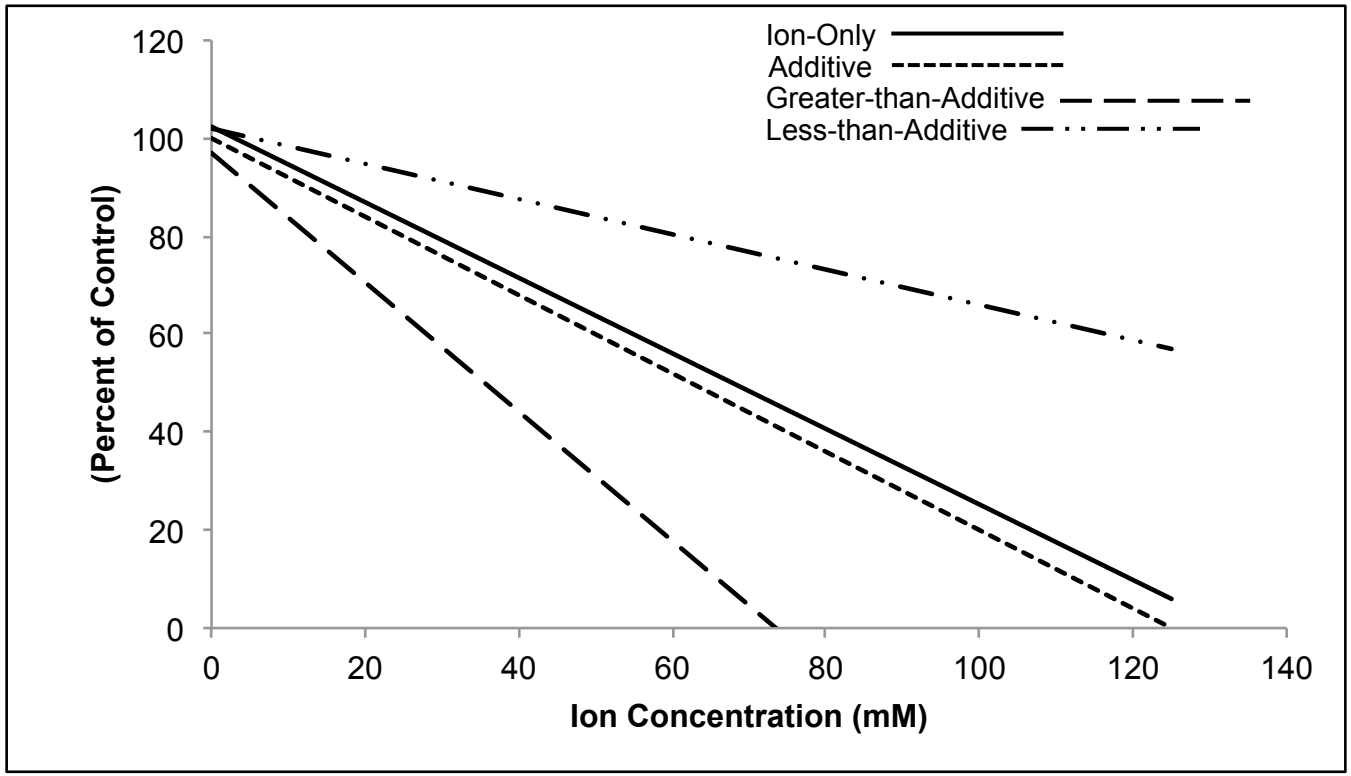

Figure 3.2. Slope analysis approach to determine contaminant mixture interactions. Results indicate additive, greater-than-additive, and less-than-additive.

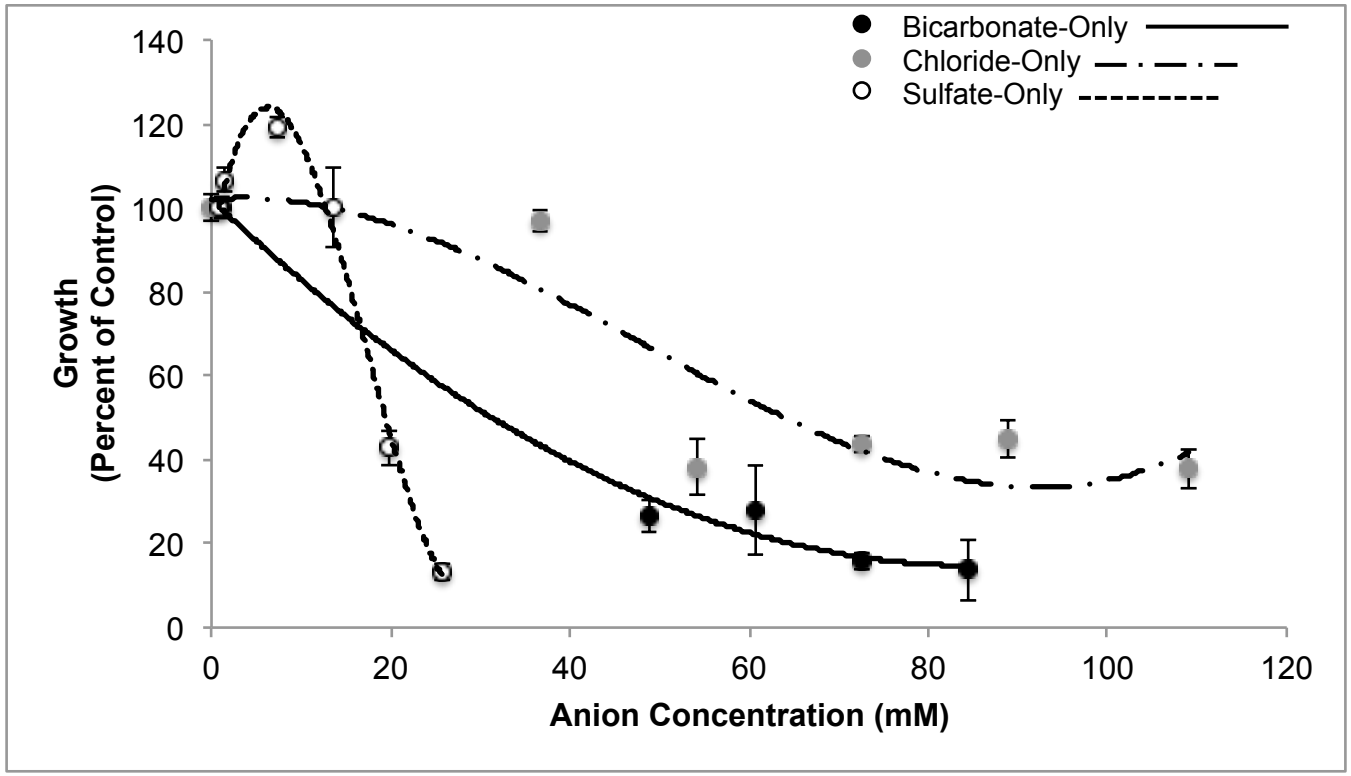

Figure 3.3. The effect of bicarbonate, chloride, and sulfate on *P. promelas* growth. Data points represent the average percent growth for each treatment (± 95% confidence intervals ($\alpha = 0.05$)).

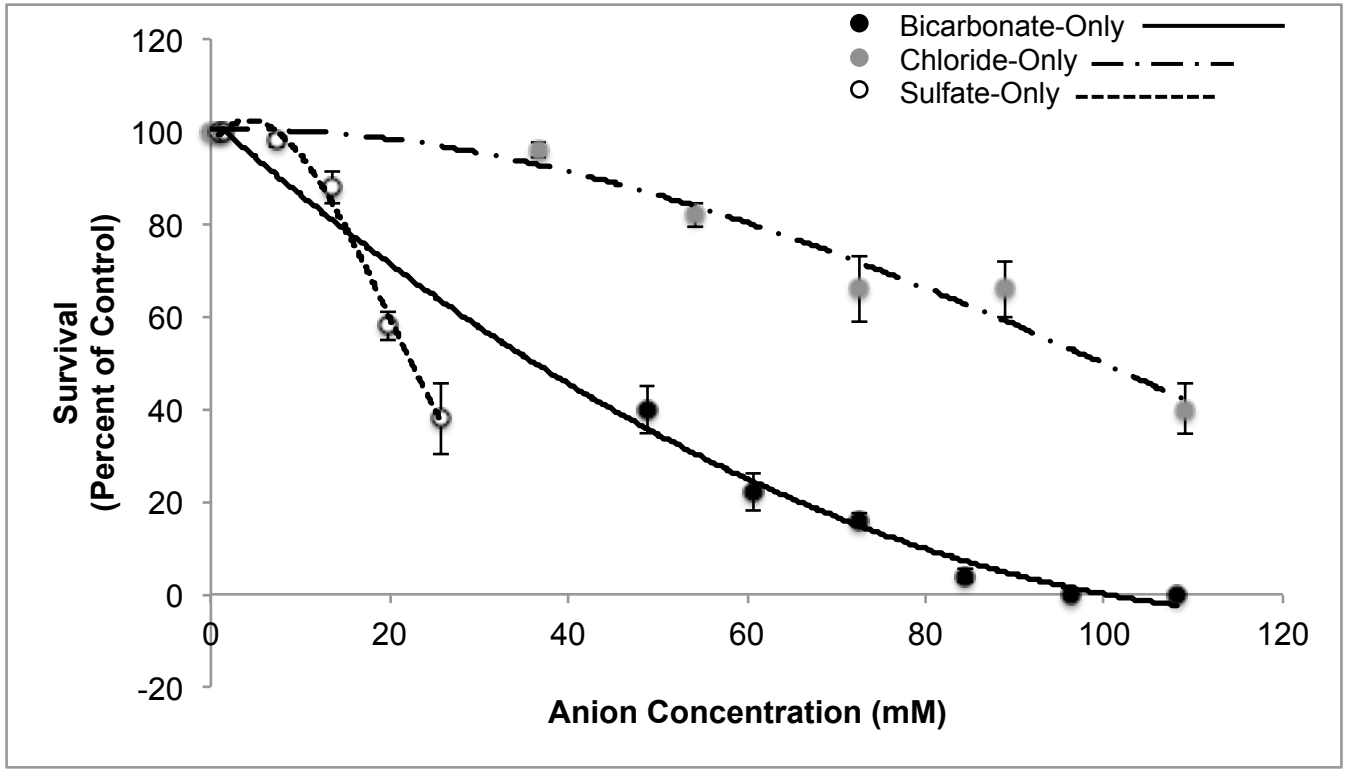

Figure 3.4. The effect of bicarbonate, chloride, and sulfate on *P. promelas* survival. Data points represent the average percent survival for each treatment (± 95% confidence intervals ($\alpha = 0.05$)).

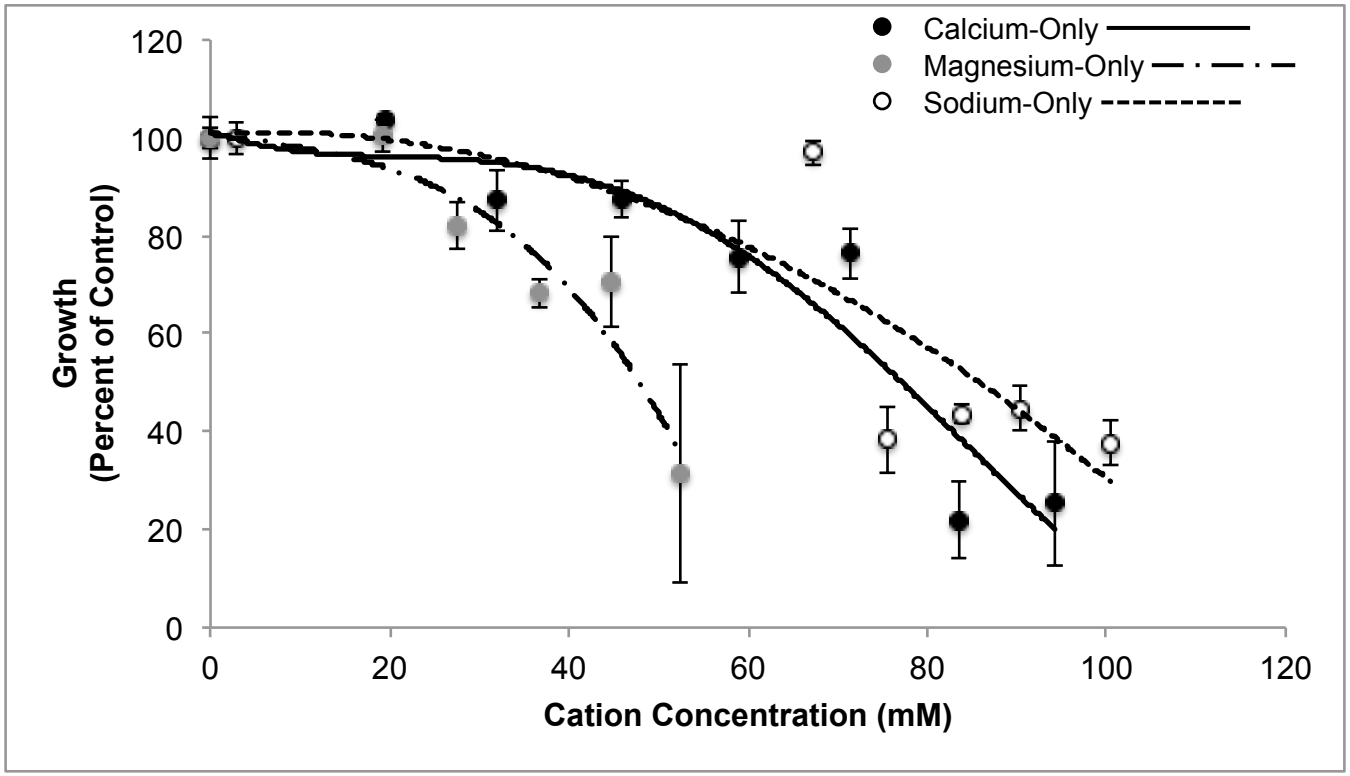

Figure 3.5. The effect of calcium, magnesium, and sodium on *P. promelas* growth. Data points represent the average percent growth for each treatment (± 95% confidence intervals ($\alpha = 0.05$)).

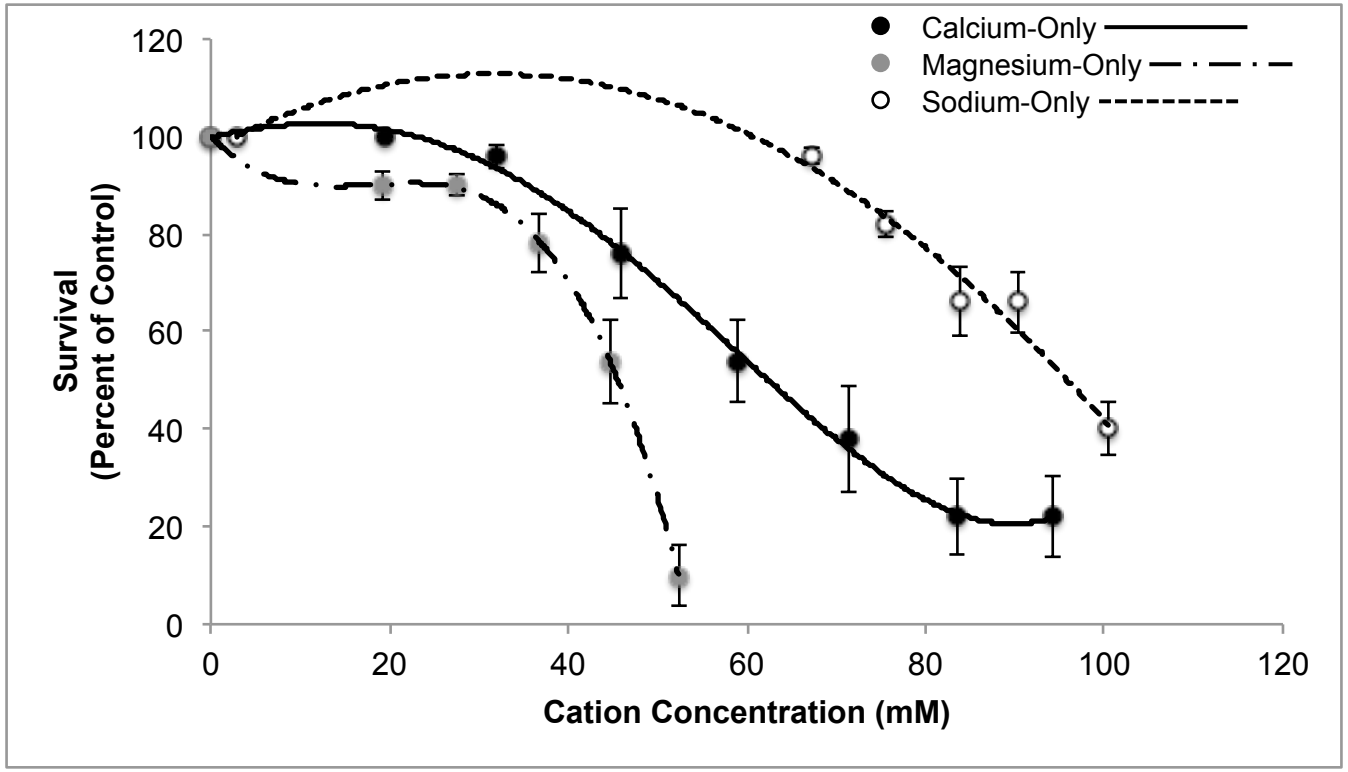

Figure 3.6. The effect of calcium, magnesium, and sodium on *P. promelas* survival. Data points represent the average percent survival for each treatment (± 95% confidence intervals ($\alpha = 0.05$)).

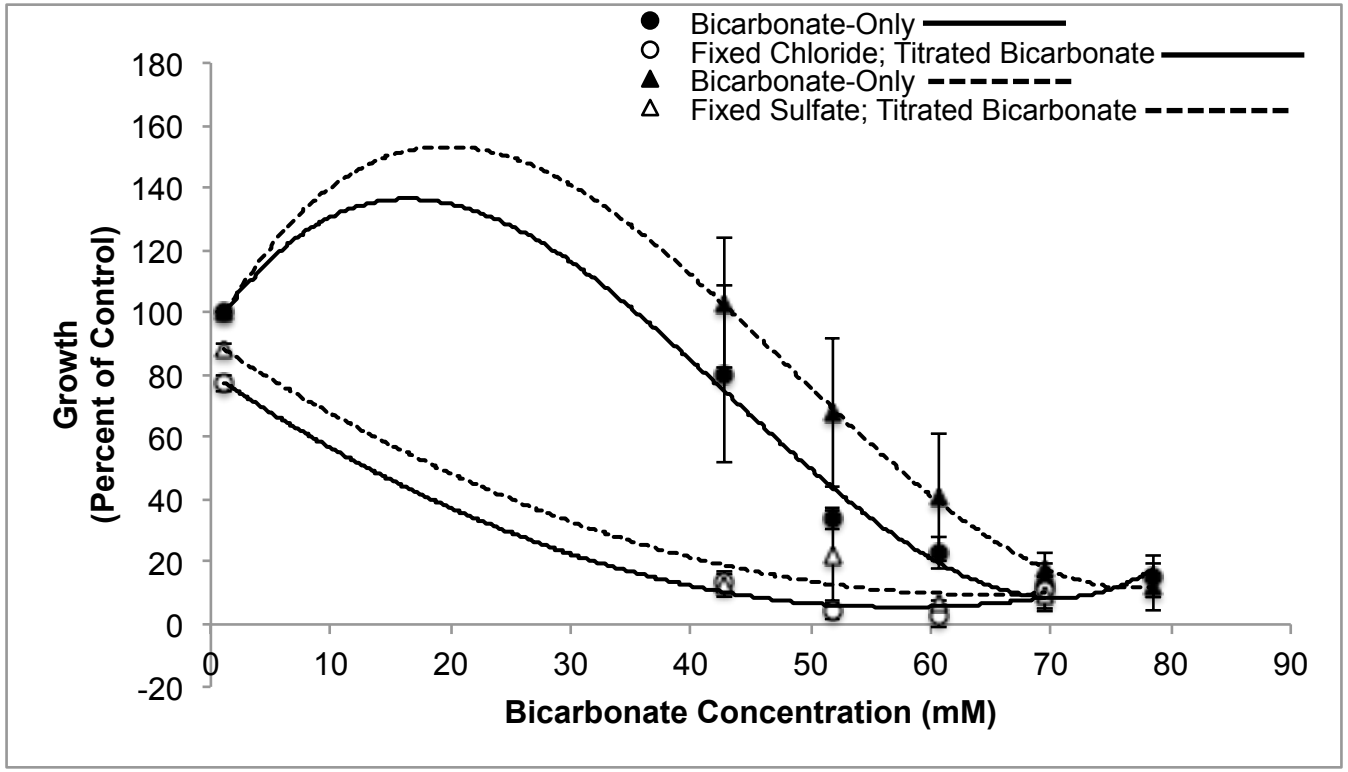

Figure 3.7. The effect of chloride and sulfate on bicarbonate toxicity on *P. promelas* growth. Individual data points denote average percent growth for each treatment (± 95% confidence intervals ($\alpha = 0.05$)).

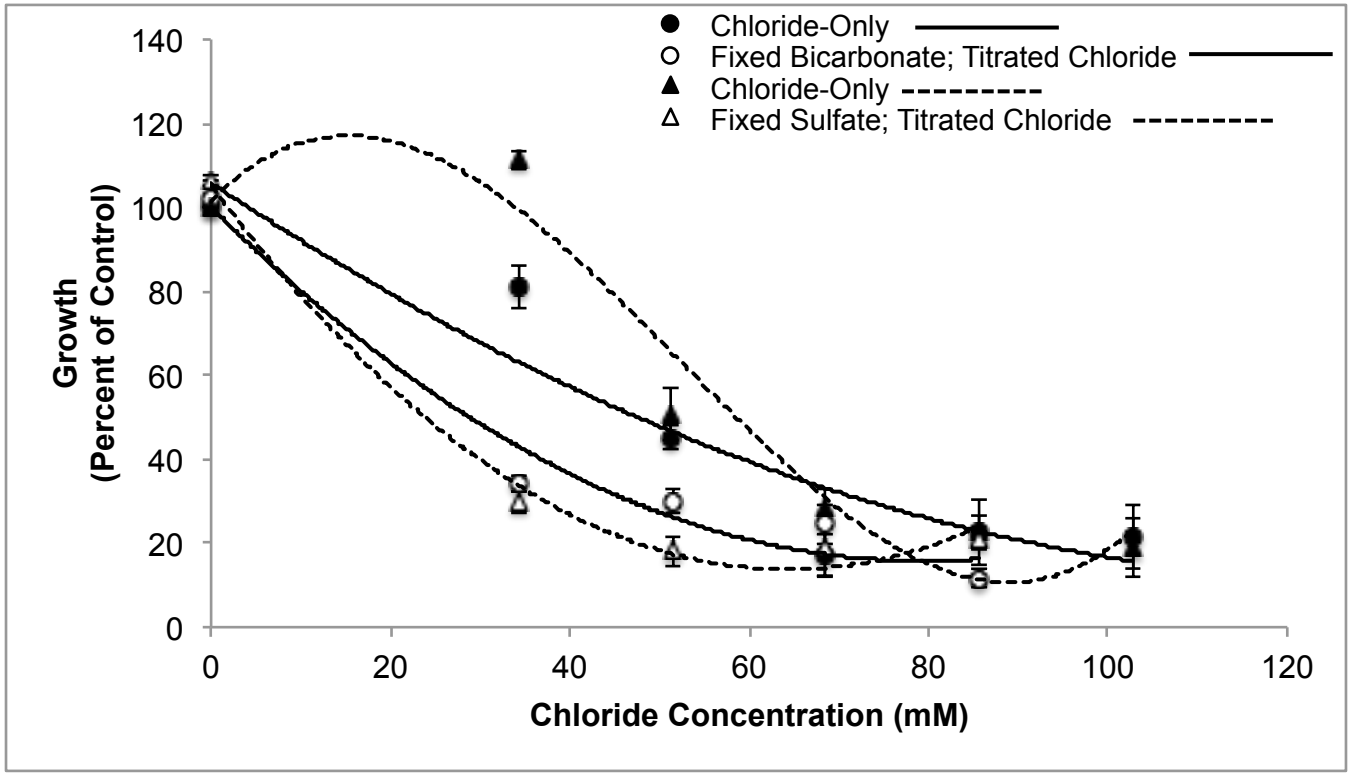

Figure 3.8. The effect of bicarbonate and sulfate on chloride toxicity on *P. promelas* growth. Individual data points denote average percent growth for each treatment (± 95% confidence intervals ($\alpha = 0.05$)).

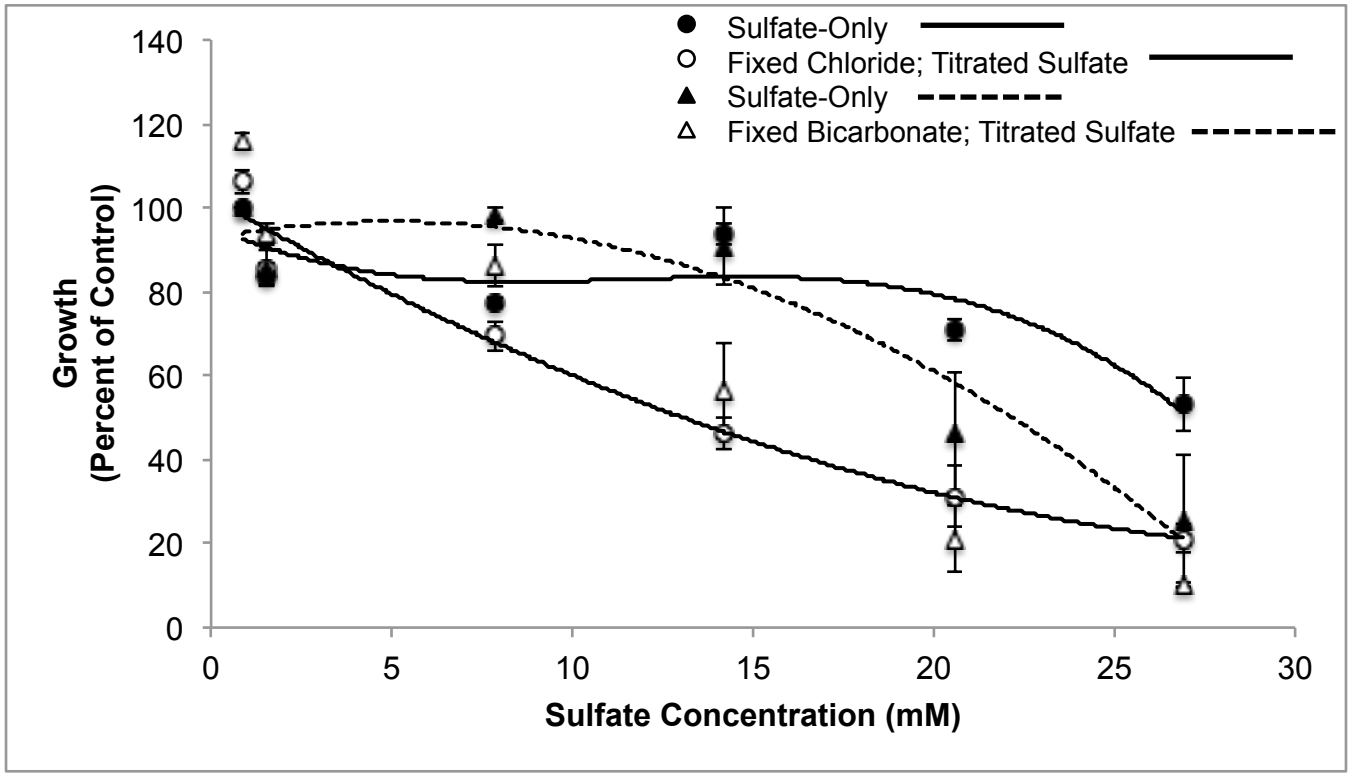

Figure 3.9. The effect of chloride and bicarbonate on sulfate toxicity on *P. promelas* growth. Individual data points denote average percent growth for each treatment (± 95% confidence intervals ($\alpha = 0.05$)).

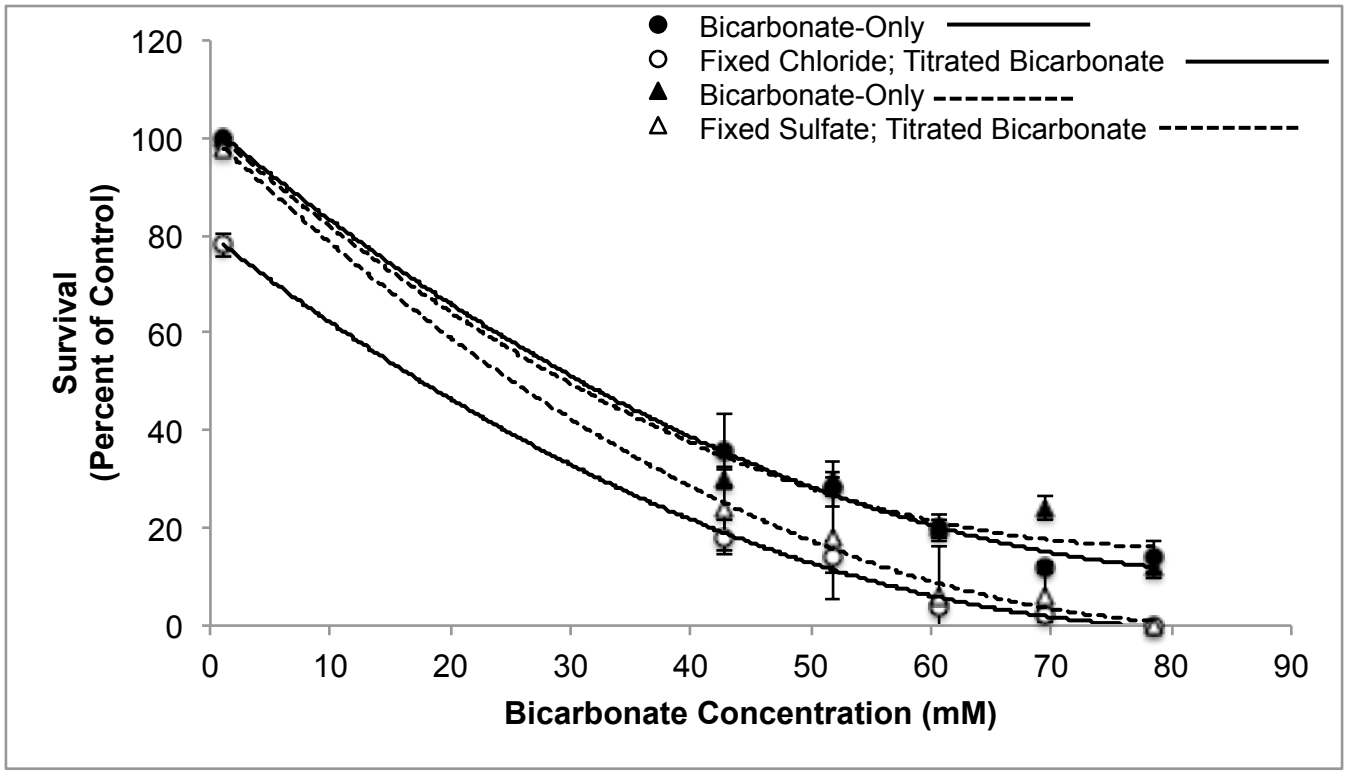

Figure 3.10. The effect of chloride and sulfate on bicarbonate toxicity on *P. promelas* survival. Individual data points denote average percent survival for each treatment (± 95% confidence intervals ($\alpha = 0.05$)).

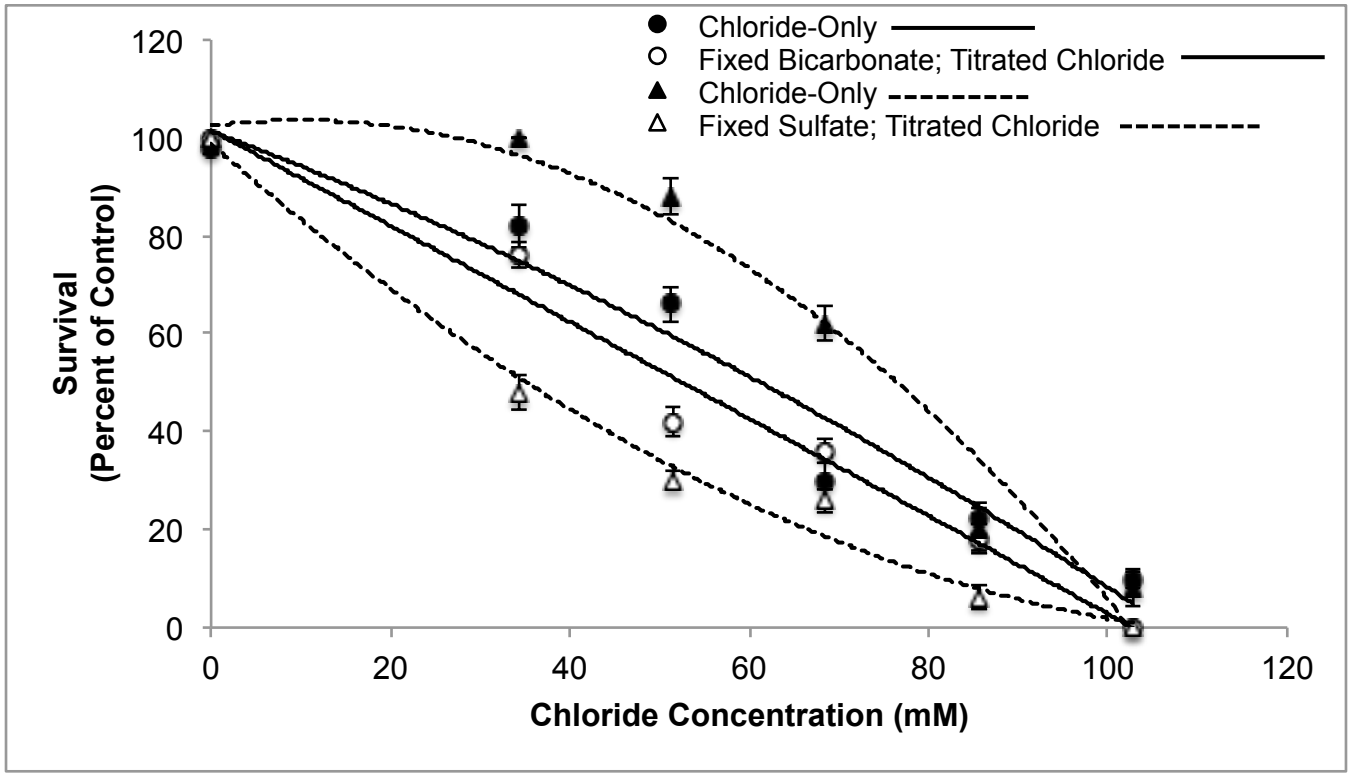

Figure 3.11. The effect of bicarbonate and sulfate on chloride toxicity on *P. promelas* survival. Individual data points denote average percent survival for each treatment (± 95% confidence intervals ($\alpha = 0.05$)).

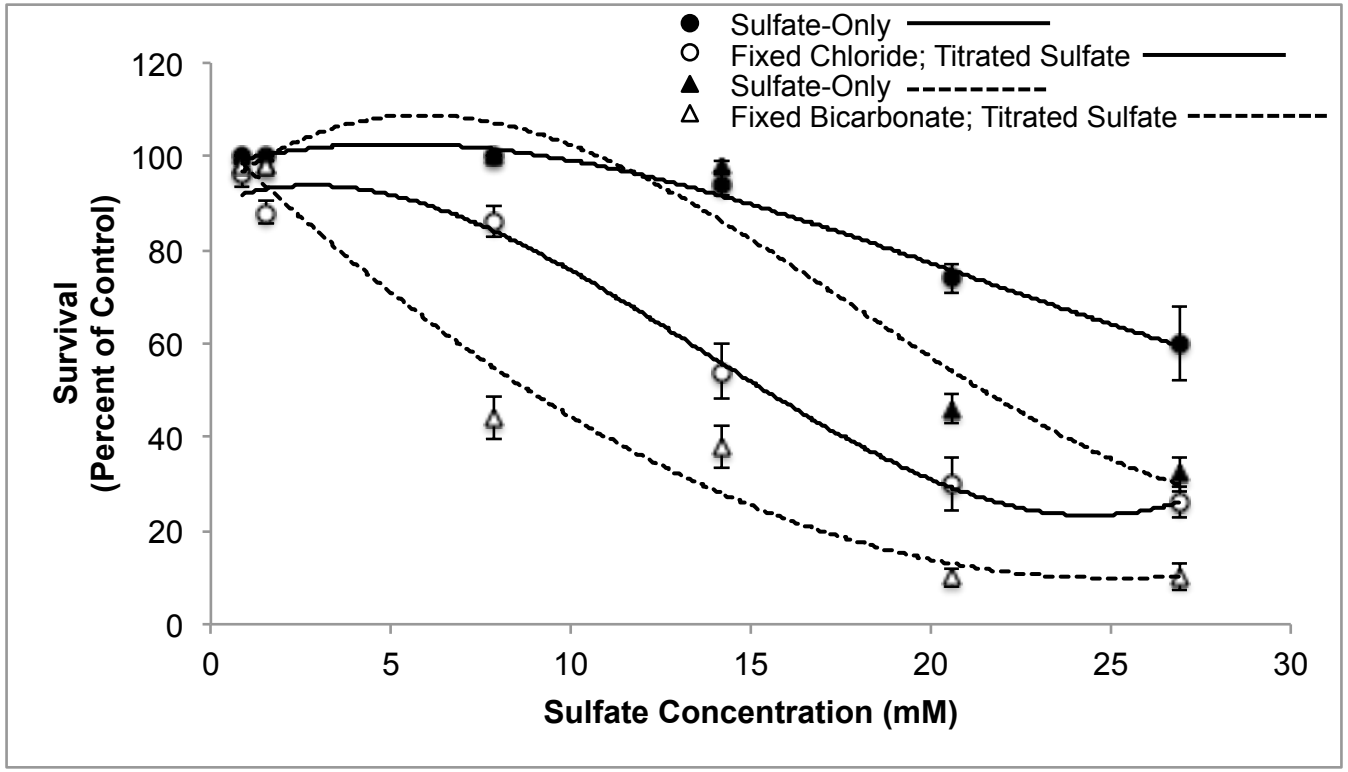

Figure 3.12. The effect of chloride and bicarbonate on sulfate toxicity on *P. promelas* survival. Individual data points denote average percent survival for each treatment (± 95% confidence intervals ($\alpha = 0.05$)).

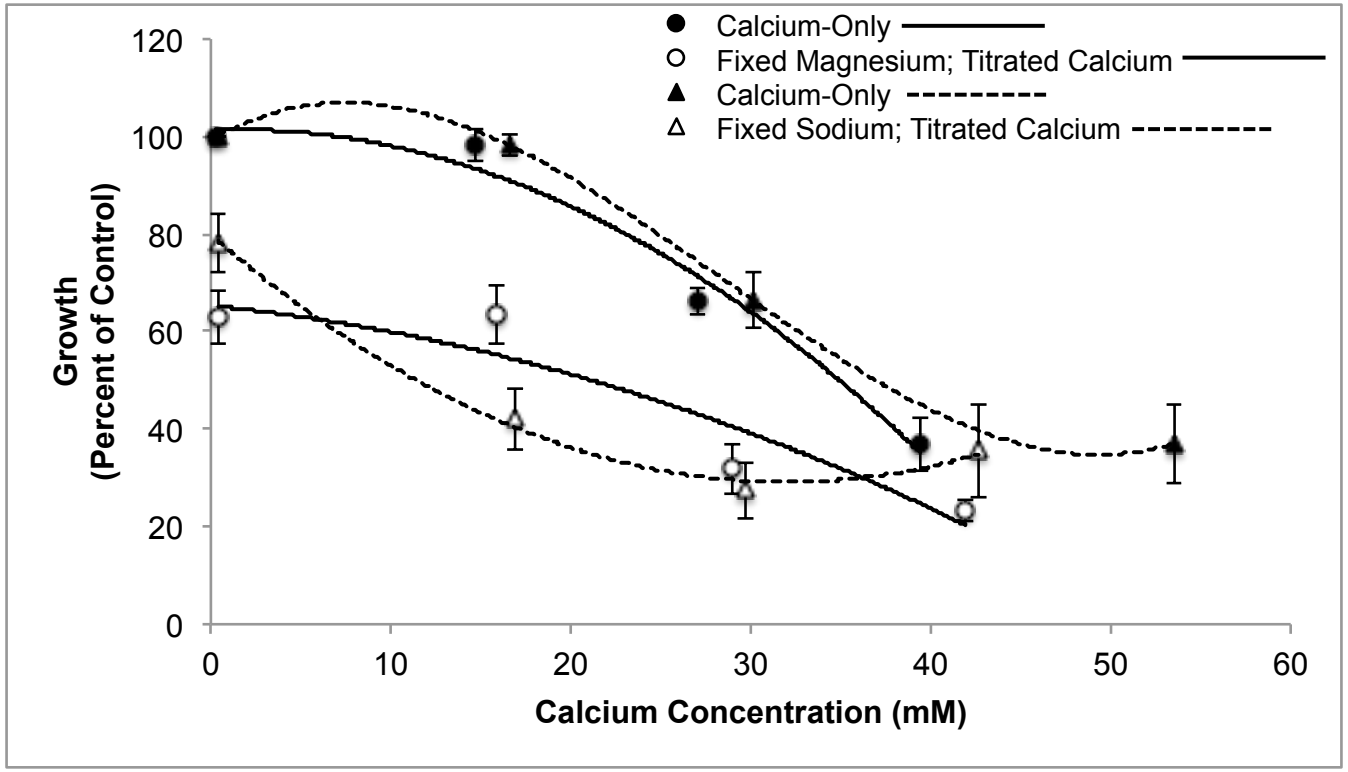

Figure 3.13. The effect of magnesium and sodium on calcium toxicity on *P. promelas* growth. Individual data points denote average percent growth for each treatment (± 95% confidence intervals ($\alpha = 0.05$)).

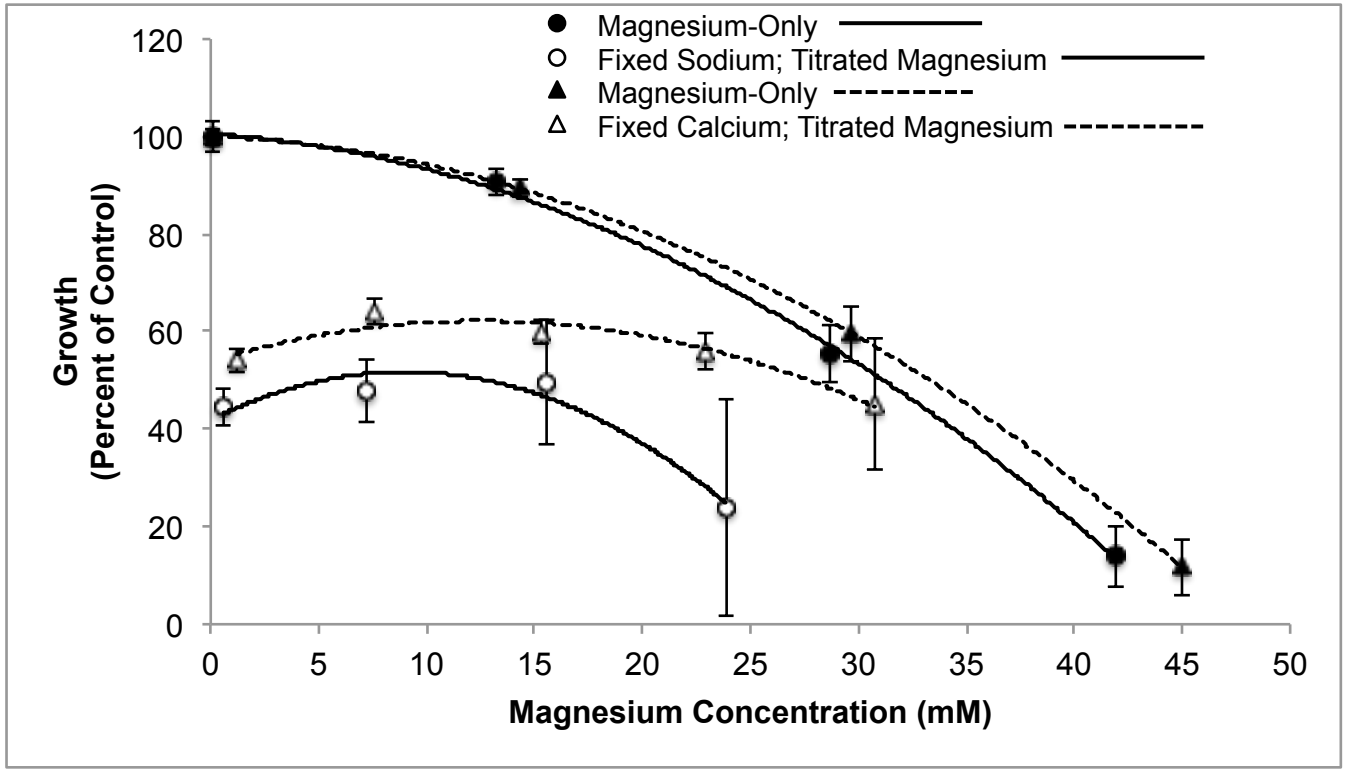

Figure 3.14. The effect of sodium and calcium on magnesium toxicity on *P. promelas* growth. Individual data points denote average percent growth for each treatment (± 95% confidence intervals ($\alpha = 0.05$)).

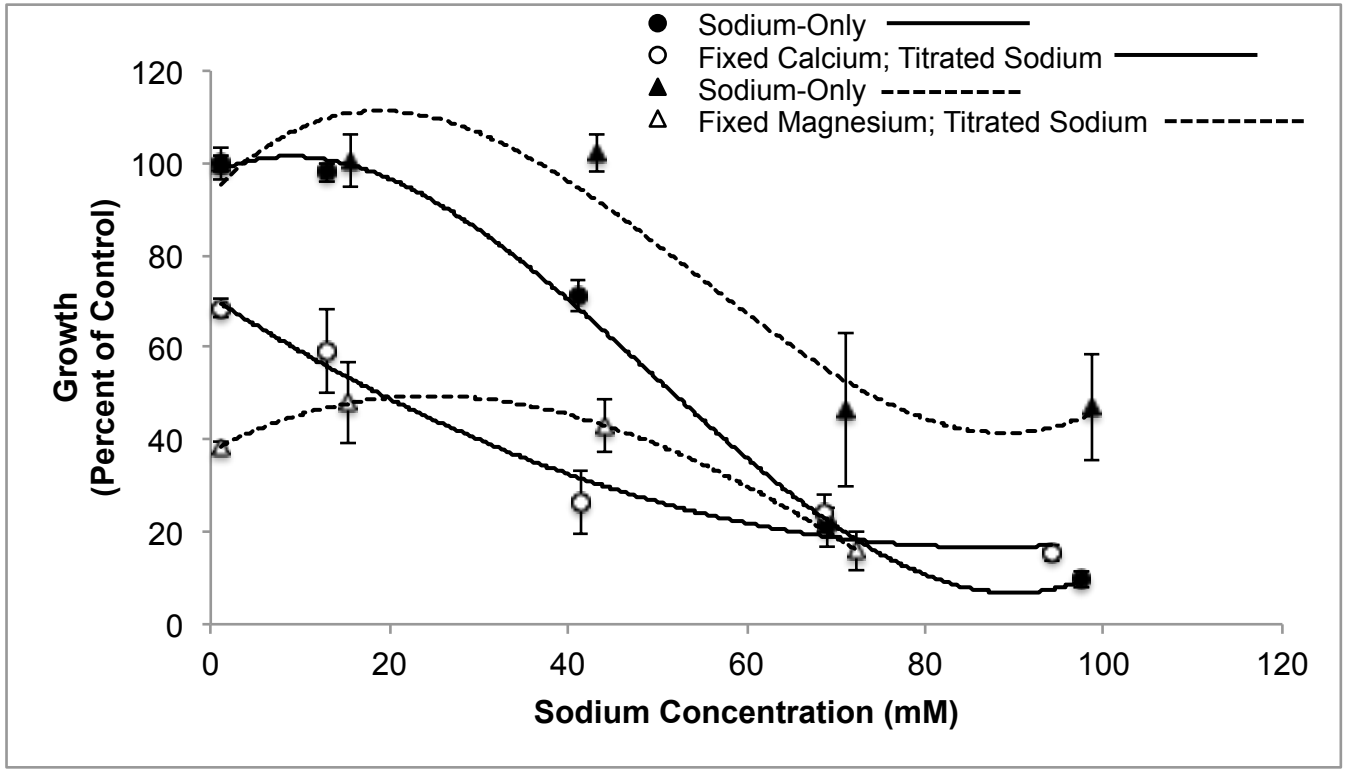

Figure 3.15. The effect of calcium and magnesium on sodium toxicity on *P. promelas* growth. Individual data points denote average percent growth for each treatment (± 95% confidence intervals ($\alpha = 0.05$)).

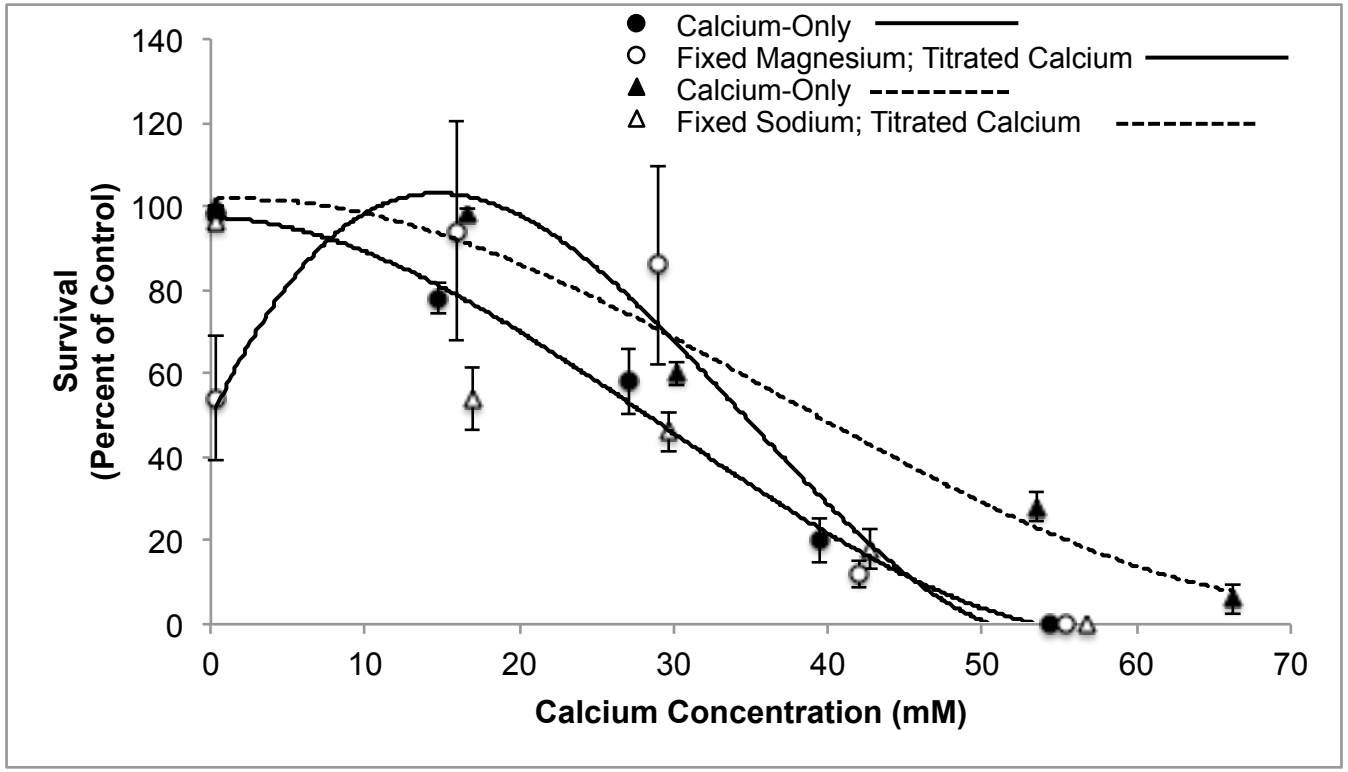

Figure 3.16. The effect of magnesium and sodium on calcium toxicity on *P. promelas* survival. Individual data points denote average percent survival for each treatment (± 95% confidence intervals ($\alpha = 0.05$)).

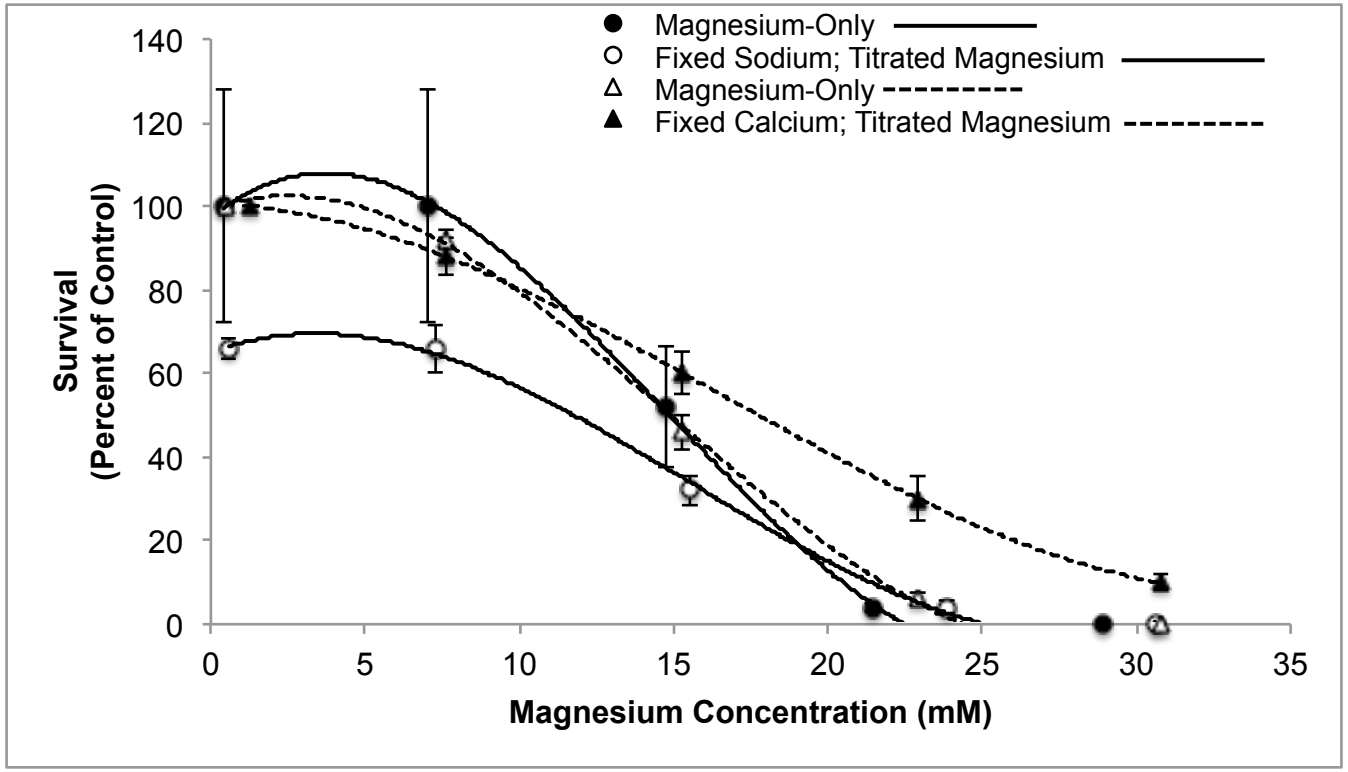

Figure 3.17. The effect of sodium and calcium on magnesium toxicity on *P. promelas* survival. Individual data points denote average percent survival for each treatment (± 95% confidence intervals ($\alpha = 0.05$)).

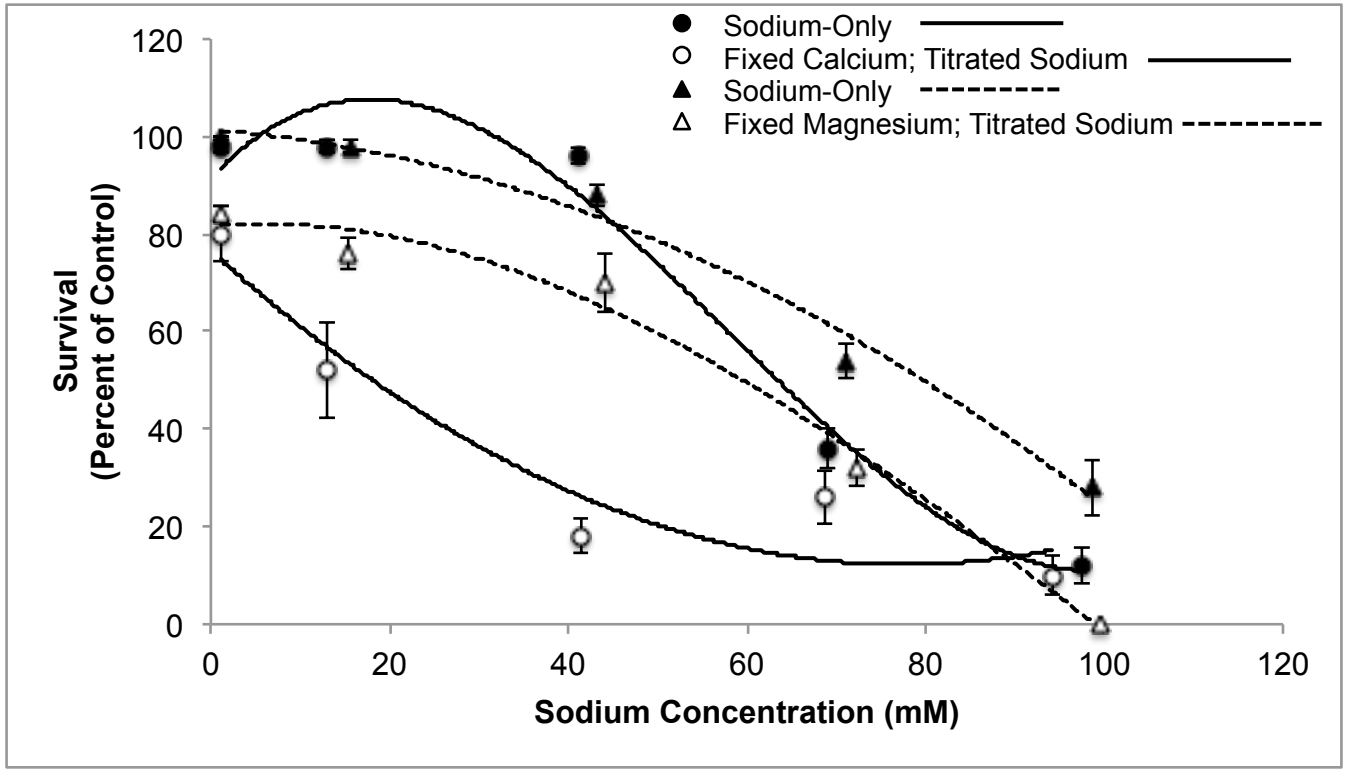

Figure 3.18. The effect of calcium and magnesium on sodium toxicity on *P. promelas* survival. Individual data points denote average percent survival for each treatment (± 95% confidence intervals ($\alpha = 0.05$)).

Table 3.1. EC_{50} values for growth and LC_{50} values for survival in *P. promelas*. Estimations derived for both conductivity and specific ion concentration[a].

Ion	Conductivity (µS/cm)		Ion Concentration (mM)	
	EC_{50}	LC_{50}	EC_{50}	LC_{50}
Chloride	5354 [c] (4981, 5728)	10158 (9538, 10777)	50.7 [d] (47.1, 54.4)	100.4 (90.9, 109.8)
Sulfate	3841 [b] (3622, 4059)	4258 (3799, 4718)	19.2 [a] (17.9, 20.5)	21.6 (19.0, 24.2)
Bicarbonate	3029 [a] (2663, 4010	3391 (2858, 3925)	33.6 [c] (30.9, 36.2)	41.8 (34.5, 49.2)
Calcium	8224 [d] (7554, 8893)	6660 (5739, 7581)	38.7 [c] (35.5, 41.8)	31.0 (26.5, 35.7)
Magnesium	4313 [b] (3916, 4709)	4072 (3790, 4353)	24.5 [b] (23.7, 25.70	23.3 (21.4, 25.3)
Sodium	5355 [c] (4981, 5728)	10078 (9265, 10890)	73.9 [d] (72.2, 75.6)	95.6 (91.0, 100.2)

[a] Parentheses indicate 95% confidence intervals for each EC_{50} and LC_{50} ($\alpha = 0.05$).

Letters next to EC_{50} values denote significant differences, determined by non-overlapping confidence intervals. Differences were determined for the two measurements independently.

Table 3.2. The effect of sulfate and chloride on bicarbonate toxicity to *P. promelas*[a,b,c,d].

	Bicarbonate Binary Mixtures				
	Slope (mM)	**EC_{50}**	**R^2**	**P-Value**	**Effect**
Bicarbonate-Only	-3.17	57.3 (51.9, 62.7)	0.637		
Fixed Sulfate; Titrated Bicarbonate	-1.81	22.3 (19.9, 24.8)	0.903	0.099	Additive
Bicarbonate-Only	-5.18	48.6 (43.5, 53.8)	0.719		
Fixed Chloride; Titrated Bicarbonate	-1.53	18.8 (15.8, 21.9)	0.977	0.123	Additive
	Slope (mM)	**LC_{50}**	**R^2**	**P-Value**	**Effect**
Bicarbonate-Only	-1.83	26.0 (19.5, 32.7)	0.929		
Fixed Sulfate; Titrated Bicarbonate	-1.78	28.2 (24.1, 32.2)	0.918	0.613	Additive
Bicarbonate-Only	-1.54	33.7 (25.2, 42.2)	0.859		
Fixed Chloride; Titrated Bicarbonate	-1.44	20.6 (15.9, 25.3)	0.926	0.772	Additive

[a] Parenthesis indicate 95% confidence intervals ($\alpha = 0.05$)

[b] R^2 values were derived from the best-fit curve for each concentration-response line.

[c] *p* values indicate significant differences between bicarbonate-only slope, and the bicarbonate mixture slope.

[d] ANCOVA was performed for effect on growth (EC_{50}) and survival (LC_{50}).

Table 3.3. The effect of bicarbonate and sulfate on chloride toxicity to *P. promelas*[a,b,c,d].

	Chloride Binary Mixtures				
	Slope (mM)	**EC_{50}**	**R^2**	**P-Value**	**Effect**
Chloride-Only	-1.88	48.8 (44.8, 51.8)	0.924		
Fixed Bicarbonate; Titrated Chloride	-1.98	26.4 (22.4, 30.3)	0.924	0.751	Additive
Chloride-Only	-1.88	48.4 (44.8, 51.8)	0.924		
Fixed Sulfate; Titrated Chloride	-2.23	83.1 (81.3, 84.8)	0.959	0.0178*	Less-than-Additive
	Slope (mM)	**LC_{50}**	**R^2**	**P-Value**	**Effect**
Chloride-Only	-2.10	58.6 (53.2, 63.9)	0.871		
Fixed Bicarbonate; Titrated Chloride	-0.988	52.4 (49.0, 55.8)	0.935	0.0178*	Less-than-Additive
Chloride-Only	-2.10	59.7 (54.3, 65.1)	0.871		
Fixed Sulfate; Titrated Chloride	-1.05	91.8 (86.6, 96.9)	0.946	0.0497*	Less-than-Additive

[a] Parenthesis indicate 95% confidence intervals ($\alpha = 0.05$)

[b] R^2 values were derived from the best-fit curve for each concentration-response line.

[c] *p* values indicate significant differences between bicarbonate-only slope, and the bicarbonate mixture slope.

[d] ANCOVA was performed for effect on growth (EC_{50}) and survival (LC_{50}).

Table 3.4. The effect of chloride and bicarbonate on sulfate toxicity to *P. promelas*[a,b,c,d].

	Sulfate Binary Mixtures				
	Slope (mM)	**EC_{50}**	**R^2**	**P-Value**	**Effect**
Sulfate-Only	-3.20	27.6 (24.6, 30.9)	0.521		
Fixed Chloride; Titrated Sulfate	-3.12	13.0 (10.5, 15.5)	0.876	0.927	Additive
Sulfate-Only	-7.03	20.1 (17.8, 22.5)	0.619		
Fixed Bicarbonate; Titrated Sulfate	-5.12	15.0 (13.0, 17.0)	0.873	0.511	Additive
	Slope (mM)	**LC^{50}**	**R^2**	**P-Value**	**Effect**
Sulfate-Only	-3.15	28.1 (21.0, 35.3)	0.642		
Fixed Chloride; Titrated Sulfate	-4.42	14.8 (12.8, 16.9)	0.817	0.469	Additive
Sulfate-Only	-8.21	20.1 (19.4, 20.9)	0.947		
Fixed Bicarbonate; Titrated Sulfate	-8.52	8.05 (5.79, 10.3)	0.907	0.836	Additive

[a] Parenthesis indicate 95% confidence intervals ($\alpha = 0.05$)

[b] R^2 values were derived from the best-fit curve for each concentration-response line.

[c] *p* values indicate significant differences between bicarbonate-only slope, and the bicarbonate mixture slope.

[d] ANCOVA was performed for effect on growth (EC_{50}) and survival (LC_{50}).

Table 3.5. The effect of magnesium and sodium on calcium toxicity to *P. promelas*[a,b,c,d].

	Calcium Binary Mixtures				
	Slope (mM)	**EC_{50}**	**R^2**	**P-Value**	**Effect**
Calcium-Only	-2.49	30.9 (26.0, 35.9)	0.926		
Fixed Magnesium; Titrated Calcium	-2.49	21.4 (24.6, 32.7)	0.564	0.921	Additive
Calcium-Only	-1.85	45.4 (39.9, 50.9)	0.874		
Fixed Sodium; Titrated Calcium	-2.18	13.3 (6.99, 19.6)	0.622	0.690	Additive
	Slope (mM)	**LC_{50}**	**R^2**	**P-Value**	**Effect**
Calcium-Only	-3.06	28.7 (24.6, 32.7)	0.858		
Fixed Magnesium; Titrated Calcium	-5.69	37.2 (29.3, 45.1)	0.648	0.086	Additive
Calcium-Only	-1.84	38.7 (33.8, 43.7)	0.925		
Fixed Sodium; Titrated Calcium	-1.65	24.9 (20.9, 28.9)	0.816	0.521	Additive

[a] Parenthesis indicate 95% confidence intervals ($\alpha = 0.05$)

[b] R^2 values were derived from the best-fit curve for each concentration-response line.

[c] *p* values indicate significant differences between bicarbonate-only slope, and the bicarbonate mixture slope.

[d] ANCOVA was performed for effect on growth (EC_{50}) and survival (LC_{50}).

Table 3.6. The effect of sodium and calcium on magnesium toxicity to *P. promelas*[a,b,c,d].

	Magnesium Binary Mixtures				
	Slope (mM)	**EC_{50}**	**R^2**	**P-Value**	**Effect**
Magnesium-Only	-5.14	15.6 (13.0, 17.4)	0.885		
Fixed Sodium; Titrated Magnesium	-3.10	15.4 (8.42, 22.5)	0.149	0.315	Additive
Magnesium-Only	-6.26	16.9 (15.2, 18.7)	0.937		
Fixed Calcium; Titrated Magnesium	-0.619	52.1 (40.1, 64.2)	0.113	0.009*	Less-than-Additive
	Slope (mM)	**LC_{50}**	**R_2**	**P-Value**	**Effect**
Magnesium-Only	-6.67	14.9 (14.3, 15.5)	0.968		
Fixed Sodium; Titrated Magnesium	-3.74	12.8 (8.54, 17.1)	0.877	0.0005*	Less-than-Additive
Magnesium-Only	-5.61	14.8 (13.9, 15.6)	0.971		
Fixed Calcium; Titrated Magnesium	-3.78	17.7 (15.4, 20.1)	0.876	0.0363*	Less-than-Additive

[a] Parenthesis indicate 95% confidence intervals ($\alpha = 0.05$)

[b] R^2 values were derived from the best-fit curve for each concentration-response line.

[c] *p* values indicate significant differences between bicarbonate-only slope, and the bicarbonate mixture slope.

[d] ANCOVA was performed for effect on growth (EC_{50}) and survival (LC_{50}).

Table 3.7. The effect of calcium and magnesium on sodium toxicity to *P. promelas*[a,b,c,d].

	Sodium Binary Mixtures				
	Slope (mM)	**EC_{50}**	**R^2**	**P-Value**	**Effect**
Sodium-Only	-1.37	51.2 (47.1, 55.2)	0.961		
Fixed Calcium; Titrated Sodium	-1.16	20.2 (8.6, 31.8)	0.745	0.582	Additive
Sodium-Only	-1.99	68.3 (54.8, 81.8)	0.577		
Fixed Magnesium; Titrated Sodium	-0.958	67.8 (51.7, 83.8)	0.368	0.211	Additive
	Slope (mM)	**LC_{50}**	**R^2**	**P-Value**	**Effect**
Sodium-Only	-2.15	62.9 (55.5, 70.4)	0.947		
Fixed Calcium; Titrated Sodium	-1.20	64.8 (59.6, 69.8)	0.569	0.171	Additive
Sodium-Only	-1.08	74.0 (66.4, 81.8)	0.878		
Fixed Magnesium; Titrated Sodium	-1.34	61.1 (53.6, 68.7)	0.879	0.529	Additive

[a] Parenthesis indicate 95% confidence intervals ($\alpha = 0.05$)

[b] R^2 values were derived from the best-fit curve for each concentration-response line.

[c] *p* values indicate significant differences between bicarbonate-only slope, and the bicarbonate mixture slope.

[d] ANCOVA was performed for effect on growth (EC_{50}) and survival (LC_{50}).

Table 3.8. Ion-only slope comparisons for growth and survival of *P. promelas*. Ion-only concentration-response lines produced for comparison during binary mixture exposures[a].

	P-Value	
	Growth	**Survival**
Bicarbonate	0.480	0.626
Chloride	0.193	0.472
Sulfate	0.154	0.0329*
Calcium	0.200	0.198
Magnesium	0.403	0.115
Sodium	0.273	0.0206*

[a] An asterisk (*) indicates significant differences between slopes.

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CHAPTER FOUR

THE CHRONIC TOXICITY OF SINGLE IONS AND BINARY ION MIXTURES OF AN INVERTEBRATE SPECIES (*C. dubia*) AND A VERTEBRATE SPECIES (*P. promelas*): A COMPARISON

Species Comparison

An initial comparison was made by plotting the relationship between effective concentrations that resulted in either a 50% decrease in growth for *P. promelas* or a 50% decrease in reproduction for *C. dubia*. The initial plot revealed a low correlation and a R-square value of only 0.485 (Figure 4.1). Although the R-square value was low, a positive trend was observed; as the EC_{50} value increased for *P. promelas*, it also tended to increase for *C. dubia*. The estimates were then separated into EC_{50} values derived from single-ion exposures, and those derived from binary mixture exposures (Figure 4.2). A linear regression for single-ion exposures produced a much higher R-square value of 0.789, indicating a strong relationship between the ion toxicity in the two organisms. In all cases, the EC_{50} values estimated for *P. promelas* were much larger, suggesting that the vertebrate species is less sensitive to and may be better at controlling the influx of elevated ions than the invertebrate species. These results corroborate similarly with those previously published for acute ion toxicity (Mount et al., 1997; Kennedy et al., 2003; Wang et al., 2016). In the present study, the smallest difference among EC_{50} estimates between the two organisms occurred with sulfate, whereas the largest difference included sodium and chloride. Although the EC_{50} estimates were very different, the slopes for most ions tended to be similar, particularly for chloride, calcium, magnesium, and sodium

(Table 4.1). This similarity may indicate that even though *P. promelas* may tolerate larger ion concentrations, they respond similarly to a certain threshold as *C. dubia* with changes in ion concentration on a milli-molar basis.

Initial ion toxicity for *C. dubia* resulted in an overall toxicity gradient of $Ca^{2+} \geq Mg^{2+} \geq SO_4^{2-} > HCO_3^- > Cl^- > Na^+$. Although this was slightly different than that described for *P. promelas* ($SO_4^{2-} > Mg^{2+} > HCO_3^- \geq Ca^{2+} > Cl^- > Na^+$), there are some similarities. For example, *C. dubia* tended to be more sensitive to divalent ions (calcium, magnesium, sulfate), while less sensitive to monovalent ions (bicarbonate, chloride, sodium). Generally, this same trend holds true to *P. promelas* in that sulfate and magnesium are among the most toxic, followed by bicarbonate, which is not significantly different from calcium. Furthermore, both organisms were less affected by chloride and sodium, possibly indicating that these organisms are well adapted to managing fluctuations in the concentration of these ions.

Binary mixture effects showed the largest difference in anion responses between the two species. Similarities between *P. promelas* and *C. dubia* resulted from both titrated sulfate mixtures (additive) and fixed sulfate with titrated chloride (less-than-additive). Whereas other anion binary mixtures demonstrated additivity for *P. promelas*, results from *C. dubia* exposures revealed that titrated bicarbonate mixtures result in additional ameliorative effects, while fixed bicarbonate with titrated chloride demonstrated an enhanced toxic response (greater-than-additive) (Table 4.2).

Unlike anion results, cation binary mixtures results were similar for both species (Table 4.3). All cation binary mixtures resulted in additivity, with the exception of fixed

calcium with titrated magnesium, which resulted in a less-than-additive response. These similarities indicate that sub-lethal mixtures of calcium, magnesium, and sodium have the same effects on *C. dubia* reproduction as they do for *P. promelas* growth.

Although ion-only EC_{50} values showed a strong correlation between the two species, a plot of binary mixture EC_{50} values did not (R^2: 0.448)(Figure 4.2). Further separating these EC_{50} values into cation and anion binary mixture results still did not give rise to any definite trends (Figure 4.3). Because *C. dubia* and *P. promelas* presented similar trends in toxicity with regards to cation binary mixtures, elucidating trends through effective concentrations may not be the most suitable. For this reason, determining species response trends based on concentration-response slopes for each ion and ion mixture may have better correlations. Plots of concentration-response slopes for each species were plotted initially in one plot (Figure 4.4). A distinct trend, however, was not immediately apparent. Further separation into single-ion and binary mixture slopes, as well as cation and anion binary mixture slopes did not reveal any additional information (Figure 4.5; Figure 4.6).

The internal environment of freshwater organisms is hyperosmotic to the surrounding media, meaning that the external environment contains a much lower ion concentration than the organism itself. Naturally, ions want to move from an area of abundance to an area of insufficiency, which creates a problem for these hyperosmotic organisms. Freshwater organisms must utilize active transport of ions across the gill, intestinal tract, and kidney in order to maintain the proper ion ratios needed for cellular functions. The gills of freshwater vertebrate species, fish in particular, are comprised of

mitrochondria-rich (MR) cells that possess a series of ion channels, ion exchangers, and antiporters that aid in ion uptake (Perry et al., 2003). The gill structure of fish has been studied extensively (Perry, 1997; Perry and Fryer, 1997; Goss et al., 1998; Perry et al., 2003; Evans et al., 2005; Eddy, 2006). However, less is understood about the particular structure and function of the *Daphnia* gill. Epithelial cells within the gills of *Daphnia* species are comprised of dark cells, which contain abundant mitochondria, and light cells, which contain fewer mitochondria. For the most part, the dark cell types are believed to be primarily responsible for ionoregualtion within the *Cladoceran* order (Kikuchi, 1983). The dark cell types function by actively absorbing Na^+ and Cl^- from their surrounding environment, but the presence of more intricate channels and ion exchangers is less understood (El-Deeb Ghazy et al., 2009). One distinctly characteristic apparatus of *Daphnia* is their shell, or carapace. The carapace has been linked to offering protection from ion changes in the outside environment by providing both an impermeable surface around the body, as well as actively participating in ionoregulation (Ebert, 2005; El-Deeb Ghazy et al., 2009). Furthermore, *C. dubia* have open circulatory systems, where hemolymph is allowed to flow freely throughout the body cavity (Ebert, 2005). This is different from their vertebrate counterparts, where blood is compartmentalized.

Most ion combinations resulted in an additive interaction for both species. Additivity was concluded if the concentration-response lines produced statistically similar slopes. Comparable concentration-response curves have been suggested to indicate similar modes-of-action or mechanisms of toxicity between compounds (van der Geest et al., 2000).

Organisms utilize energy to maintain the biological functions necessary to ensure ecological success, through functions such as growth and reproduction. Freshwater organisms particularly rely on ionoregulation to control ion movement into and out of the body, which also requires energy in the form of adenosine triphosphate (ATP). If the ion concentration in the external media exceeds what is physiologically manageable for the organism, they may need to allocate more energy towards ionoregulation and reduce the energy for other functions needed in order to grow and reproduce. For example, it has been demonstrated in *C. dubia* that a reduction in energy linked to decreased filter feeding rates occurs following exposure to sodium sulfate (Soucek, 2007). Most ion channels and pumps require energy to transport ions against their concentration gradient, from an area of low concentration to high concentration (Perry et al., 2003). If the concentration of the external media increases to a concentration resulting in sub-lethal effects, it may indicate that the freshwater organisms are allocating more energy to aid in proper ionoregulation than for other energy dependent functions (Farag and Harper, 2014*a*). This would indicate that all ions have the same mode-of-action, and that energy deficiency is the main route of toxicity.

The fact that the toxicity data for both organisms resulted in mostly additive responses could indicate that the route of toxicity, or energy deficiency, is similar between the two freshwater species. Binary anion mixture toxicity resulted in mostly less-than-additive responses for *C. dubia*. This less-than-additive response is unusual because invertebrate species tend to have greater toxic responses compared to that of their more physiologically complex counterpart, *P. promelas*. Unfortunately, more is

known ionoregulation in freshwater fish and little is known regarding the ionoregulatory capabilities of *Cladoceran* species. In freshwater fish, carbonic anhydrase is the enzyme important in breaking down CO_2 waste into H^+ and HCO_3^- ions, which are ultimately excreted by the organisms (refer to Figure 1.1). It is then assumed that bicarbonate is exchanged for chloride on the apical membrane via a Cl^-/HCO_3^- exchanger, the action of which is controlled by the activity of a V-type ATPase located on the basolateral membrane of mitochondrion-rich cells (Piermarini and Evans, 2001). When the bicarbonate concentration in the external environment increases, it has been demonstrated that the Na^+/K^+-ATPase specific activity decreases in *P. promelas* (Farag and Harper, 2014*b*). A decrease in Na^+/K^+-ATPase activity could result in a decrease in Cl^-/HCO_3^- exchange rate. If this were to happen, carbonic anhydrase may become inhibited due to a buildup of bicarbonate ions, ultimately stopping the breakdown of CO_2 waste. Higher concentrations of CO_2 waste in the plasma of these freshwater fish may contribute to a decrease in blood pH and eventually death possibly due to hypercapnia and acidosis.

Future Predictive Modeling

Extensive work has been performed to develop predictive models of acute ion toxicity for field application purposes. After assessing the acute toxicity of over 2,900 ion combinations to *Ceriodaphnia dubia*, *Daphnia magna*, and *Pimephales promelas*, Mount et al. (1997) developed multivariate logistic regression models for each individual species. Initially, regression equations were developed for single ions pairs (i.e., Ca2+ and Cl- as CaCl2). These models advanced to incorporate either two cations and one anion (i.e., Na^+, Mg^{2+}, Cl^- as NaCl and $MgCl_2$) or one cation with two anions (i.e., Na^+,

Cl^-, SO_4^{2-} as Na_2SO_4 and NaCl). These more advanced models exhibited poor predictability, in particular for *C. dubia*. It was determined that the advanced models were underestimating the toxicity of solutions containing more than one cation, specifically for solutions with two sources of chloride. For example, similar toxicities occurred for single salt solutions containing NaCl and $CaCl_2$; however, when in combination, the toxicity of chloride decreased. These results implied that multiple cations in solution had an important effect on the overall toxicity of that solution, which was not incorporated into the model prediction equation. This discovery prompted the inclusion of a "NumCat" variable, which was simply the number of cations present. Final models produced R-square values of 0.861 for 24-hour LC_{50} values. Conversely, *P. promelas* acute toxicity estimates showed that the "NumCat" variable was not a significant factor in the prediction equations. R-squared values for *P. promelas* were between 0.767 and 0.832. Field-based testing revealed that models developed by Mount et al. (1997) performed well when observed toxicity of irrigation drain water sites was similar to predicted toxicity for *C. dubia* (Dickerson et al., 1996). This was dissimilar to reports indicating that *P. promelas* toxicity was over-predicted (Tietge et al., 1997). Since then, more advanced models have been expanded for *C. dubia* to include a greater understanding of the roles in which Ca^{2+} and Mg^{2+} play in the ameliorative effect on ion combinations, with little work being done to strengthen the predictability for *P. promelas* (Erickson et al., 2018).

In the present study, similarities between the chronic toxicity of *P. promelas* and *C. dubia* may suggest that prediction models may be built from combined species data,

rather than one model for each organism, increasing model applicability. These similarities further suggest that *P. promelas* growth may be a useful predictor of decreased *C. dubia* reproduction. Currently, mathematical models predicting acute ion toxicity have been developed mostly incorporating LC_{50} values and impacts of multiple cations. However, chronic toxicity data presented in this study demonstrate that anion toxicity may have further significance in model development. The reasons for these differences may lie in the mechanisms behind which these ions exert their sub-lethal vs. lethal effects on freshwater organisms. For that reason, incorporating physiological variables such as changes in enzymatic activity, osmotic pressure, blood pH and/or whole organism ion composition may increase the accuracy of additional models. The incorporation of these characteristics would help develop a model similar to a Physiologically Based Pharmacokinetic (PBPK) model. Characterizing the effect of elevated major ions on these aspects of biological functions would be difficult to complete on smaller organisms, such as *C. dubia*. However, because of the similarities described in the present study between *C. dubia* and *P. promelas*, the mechanistic response of *P. promelas* may be comparable to effects seen for *C. dubia*. These types of PBPK models separate the organism into multiple compartments and help understand the flow of ions through the ionoregulatory important organs within fish, such as the gill, intestinal tract, integument, and kidney.

The toxicity of major ions, as described above, is a very complex system. It has been demonstrated repeatedly that each ion individually produces its own toxic response for multiple endpoints, and multiple organisms. Ion mixtures have also been shown to

not necessarily act in a simply additive manner (Mount et al., 1997; Wang et al., 2016; Soucek et al., 2011; Soucek and Kennedy, 2005; Mount et al., 2016; Erickson et al., 2017). This implies that utilizing comprehensive parameters, such as conductivity or TDS, to predict or estimate toxicity may not be the most suitable. Additionally, strong accurate models could also reduce the need for site-specific regulations, and ultimately reduce the time and expense required for such a significant endeavor.

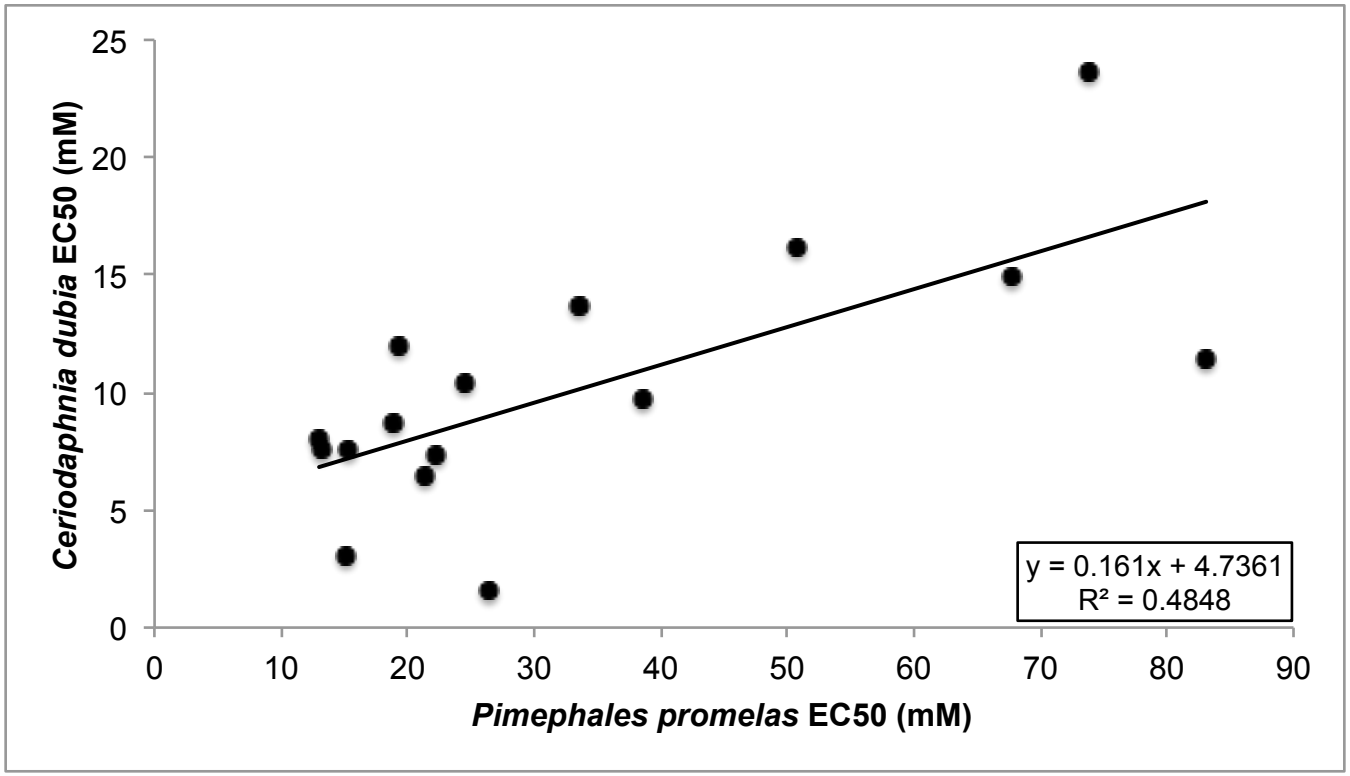

Figure 4.1. The correlation of EC_{50} values estimated for *C. dubia* reproduction and *P. promelas* growth. Data points represent EC_{50} values estimated for all ion-only and binary mixture exposures (R^2: 0.485).

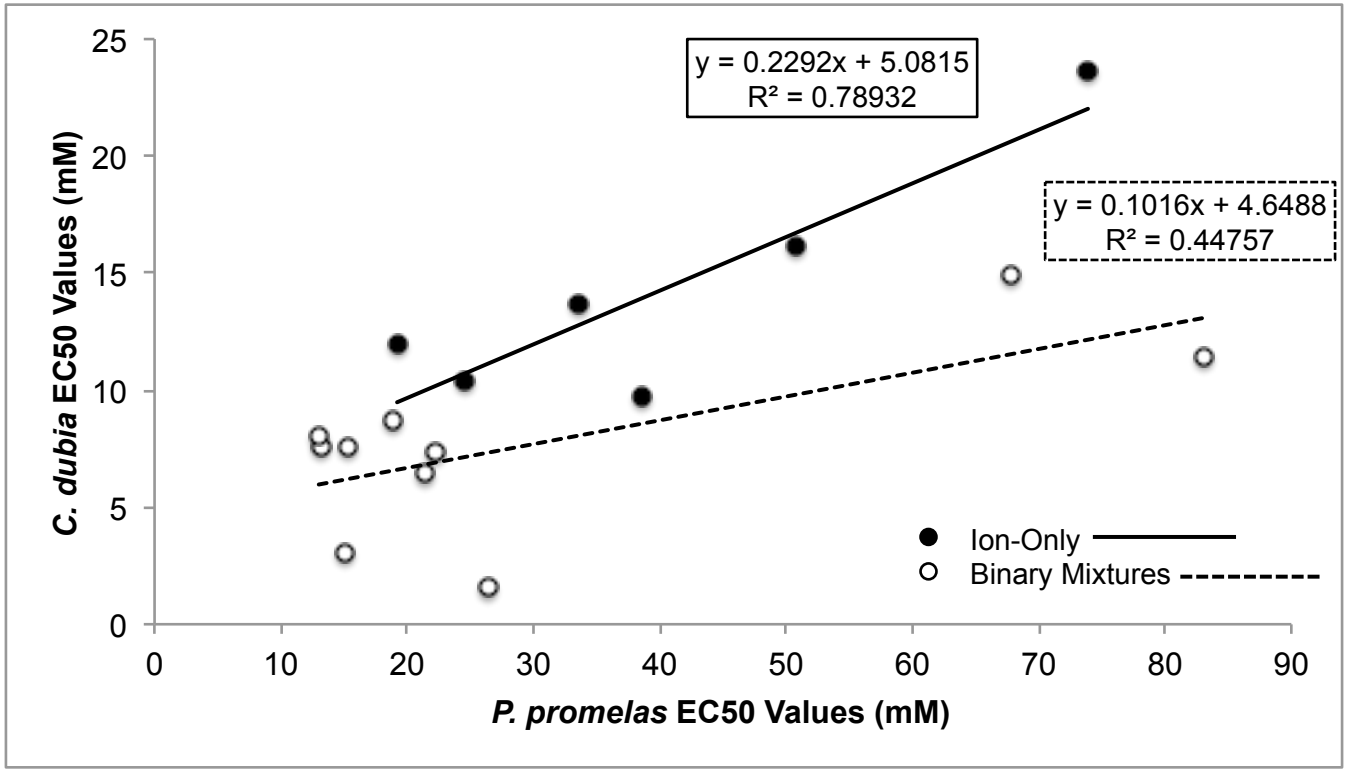

Figure 4.2. The correlation of EC_{50} values estimated for *C. dubia* reproduction and *P. promelas* growth separated by exposure type. Data points represent EC_{50} values estimated for and separated by all single-ion and binary mixture exposures. The linear regression for ion-only exposures indicates an R^2 of 0.789, whereas the binary mixture R^2 is 0.448.

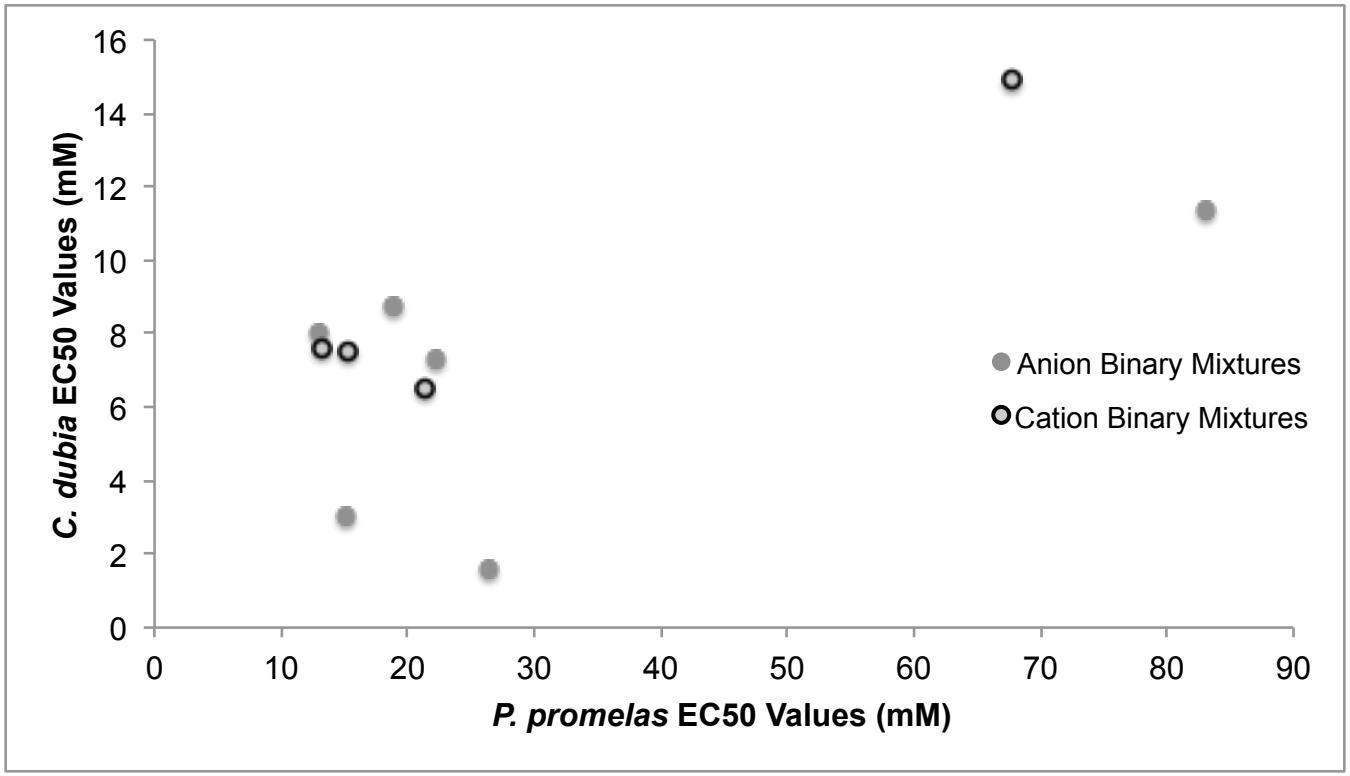

Figure 4.3. The correlation of EC_{50} values estimated for *C. dubia* reproduction and *P. promelas* growth separated by binary mixture type. Data points represent EC_{50} values estimated for and separated by cation and anion binary mixture exposures. No distinct trends between the two types of binary mixtures were noted.

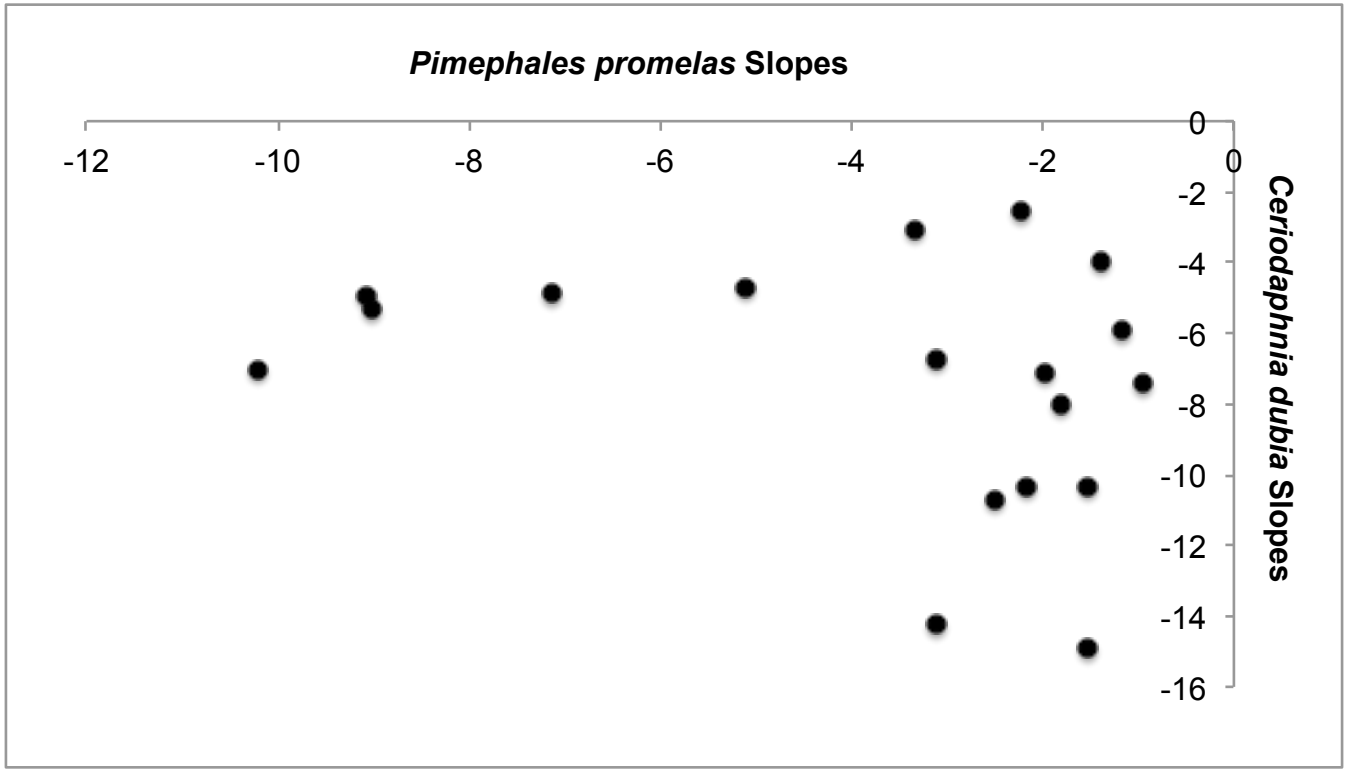

Figure 4.4. The correlation of the concentration-response slopes for *C. dubia* reproduction and *P. promelas* growth. Data points represent slopes estimated for all single-ion and binary mixture exposures.

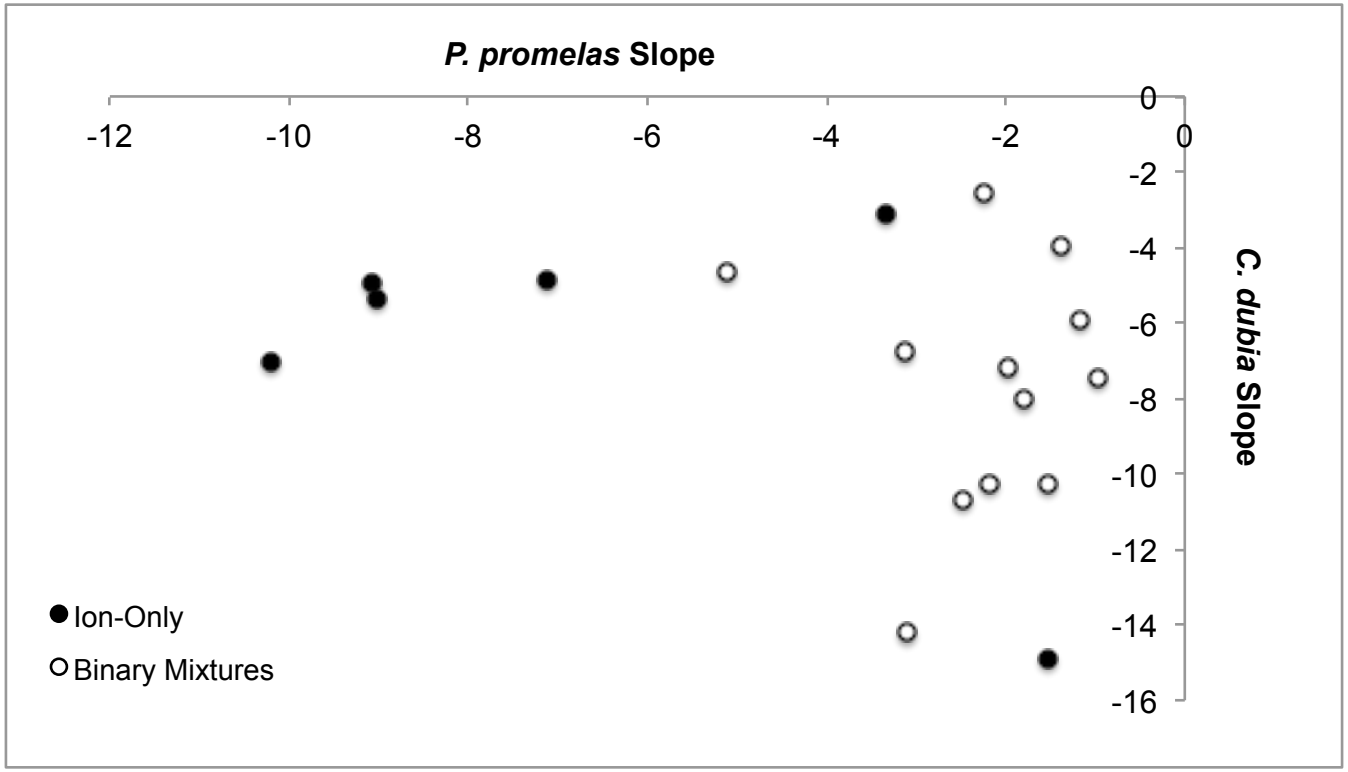

Figure 4.5. The correlation of the concentration-response slopes for *C. dubia* reproduction and *P. promelas* growth separated by exposure type. Data points represent slopes estimated for and separated by all single-ion and binary mixture exposures.

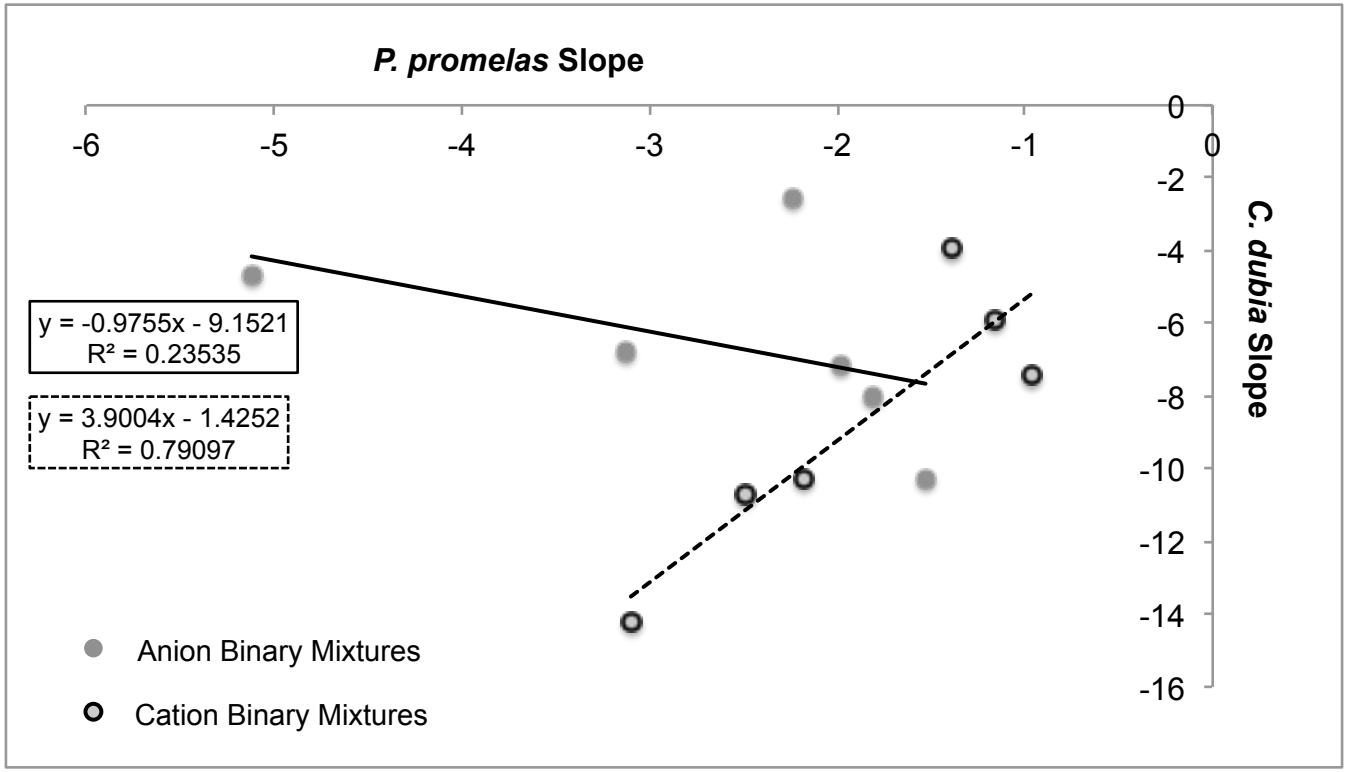

Figure 4.6. The correlation of the concentration-response slopes for *C. dubia* reproduction and *P. promelas* growth separated by binary mixture type. Data points represent slopes estimated for and separated by cation and anion binary mixture exposures. No distinct trends between the two types of binary mixtures were noted.

Table 4.1. A summary of the chronic toxicity of ion-only exposures to *C. dubia* and *P. promelas*. Results for *C. dubia* indicate effects on reproduction, while *P. promelas* results indicate effects on growth. Data are previously described in Chapter Two and Chapter Three.

	C. dubia			***P. promelas***		
Ion	**Slope**	**EC_{50} (mM)**	**R^2**	**Slope**	**EC_{50} (mM)**	**R^2**
Chloride	-3.08 (-3.41, -2.75)	16.2 (15.4, 17.1)	0.739	-3.33 (-4.51, -2.15)	50.7 (47.1, 54.4)	0.856
Sulfate	-4.94 (-5.30, -4.57)	12.0 (11.5, 12.6)	0.862	-9.08 (-13.8, -4.39)	19.2 (17.9, 20.5)	0.859
Bicarbonate	-14.9 (-17.8, -11.9)	13.7 (13.3, 14.1)	0.746	-1.54 (-1.70, -1.38)	33.6 (30.9, 36.2)	0.939
Calcium	-5.34 (-6.39, -4.27)	9.74 (8.54, 10.9)	0.855	-9.01 (-11.8, -6.19)	38.7 (35.5, 41.8)	0.742
Magnesium	-7.07 (-8.66, -5.48)	10.4 (9.50, 11.5)	0.859	-10.2 (-18.2, -2.19)	24.5 (23.7, 25.7)	0.651
Sodium	-4.89 (-6.66, -3.13)	23.6 (21.2, 26.1)	0.766	-7.12 (-9.65, -4.59)	73.9 (72.2, 75.6)	0.856

Table 4.2. A summary of the chronic toxicity of binary anion mixtures to *C. dubia* and *P. promelas*. Results for *C. dubia* indicate effects on reproduction, while *P. promelas* results indicate effects on growth. Data are previously described in Chapter Two and Chapter Three.

		C. dubia			***P. promelas***		
Fixed Ion	**Titrated Ion**	**Slope**	**P-Value**	**Effect**	**Slope**	**P-Value**	**Effect**
Bicarbonate	Chloride	-7.15 (-4.05, -1.45)	0.004*	Greater-than-Additive	-1.98 (-2.38, -1.58)	0.751	Additive
Sulfate	Chloride	-2.57 (-2.87, -2.25)	0.033*	Less-than-Additive	-2.23 (-2.44, -2.03)	0.018*	Less-than-Additive
Chloride	Sulfate	-6.75 (-9.08, -4.41)	0.057	Additive	-3.12 (-4.23, -2.02)	0.927	Additive
Bicarbonate	Sulfate	-4.69 (-5.83, -3.55)	0.701	Additive	-5.12 (-6.89, -3.39)	0.511	Additive
Chloride	Bicarbonate	-10.3 (-13.5, -7.14)	0.007*	Less-than-Additive	-1.53 (-1.76, -1.30)	0.123	Additive
Sulfate	Bicarbonate	-8.05 (-12.2, -3.91)	0.010*	Less-than-Additive	-1.81 (-2.02, -1.59)	0.099	Additive

Table 4.3. A summary of the chronic toxicity of binary cation mixtures to *C. dubia* and *P. promelas*. Results for *C. dubia* indicate effects on reproduction, while *P. promelas* results indicate effects on growth. Data are previously described in Chapter Two and Chapter Three.

		C. dubia			***P. promelas***		
Fixed Ion	**Titrated Ion**	**Slope**	**P-Value**	**Effect**	**Slope**	**P-Value**	**Effect**
Magnesium	Calcium	-10.7 (-13.7, -7.78)	0.125	Additive	-2.49 (-4.20, -0.610)	0.921	Additive
Sodium	Calcium	-10.3 (-14.2, -6.32)	0.292	Additive	-2.18 (-3.63, -0.734)	0.690	Additive
Sodium	Magnesium	-14.2 (-19.2, -9.25)	0.501	Additive	-3.10 (-7.95, 1.74)	0.315	Additive
Calcium	Magnesium	-3.97 (-7.69, -0.28)	0.004*	Less-than-Additive	-1.39 (-3.52, 0.739)	0.009*	Less-than-Additive
Calcium	Sodium	-5.92 (-9.03, -2.82)	0.660	Additive	-1.16 (-2.05, -0.285)	0.582	Additive
Magnesium	Sodium	-7.45 (-10.9, -3.93)	0.820	Additive	-0.958 (-1.56, -0.358)	0.211	Additive

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CHAPTER FIVE

THE EFFECT OF MULTI-ION EXPOSURES ON Na^+/K^+-ATPASE AND CARBONIC ANHYDRASE ACTIVITY AND RECOVERY IN *Pimephales promelas*

(fathead minnow)

Introduction

Dissolved ions have several important physiological functions within aquatic organisms. Primarily, ions create electrochemical gradients that ultimately control the movement of water and other electrolytes between the internal and external environment of these organisms (Thomas and Egee, 1998). In freshwater species, the internal ion concentration (~274 mosM) is typically greater than that of their external environment (<50 mosM) (Evans et al., 2005). As a result, freshwater species must utilize active transport to maintain a homeostatic ion balance. The primary site of active transport for the purpose of ionoregulation and acid-base regulation in freshwater fish is the gill (Gilmour and Perry, 2009). Although there are many pumps, transporters and enzymes located in the gill that are responsible for this osmotic control, carbonic anhydrase and Na^+/K^+-ATPase are essential contributors to these processes (Perry et al., 2003).

Not only is carbonic anhydrase important in acid-base regulation through the conversion of CO_2 waste into H^+ and HCO_3^- ions, it is also indirectly responsible for the exchange of Cl^- and Na^+ (Gilmour and Perry, 2009). Briefly, HCO_3^- ions are exchanged for Cl^- ions at the apical surface of the gill allowing HCO_3^- to exit the body while allowing Cl^- inside. In a similar fashion, H^+ ions are exchanged for Na^+ ions on the apical membrane. As a result, carbonic anhydrase is important in maintaining the delicate

balance between blood pH and ionic concentration (refer to Figure 1.1; Perry and Fryer, 1997). Little work has been done to describe the response of carbonic anhydrase following exposure to elevated ions, particularly freshwater fish, and in fact, many studies are contradictory. Zhanyszek and Smith (1984) suggested that carbonic anhydrase activity in the gills of coho salmon (*Oncorhynchus kisutch*), an anadromous species, was higher when adapted to saltwater conditions versus freshwater. Similarly, carbonic anhydrase activity increased also in tilapia (*Oreochromis mossambicus*) gills, a euryhaline species, when exposed to increasing salinity concentrations (Kultz et al., 1992). Alternatively, the euryaline species *Platichthys flesus* (flounder) did not show any significant changes in gill carbonic anhydrase activity between seawater and freshwater adapted fishes (Mashiter KE, MRJ Morgan, 1975).

Na^+/K^+-ATPase, being basolaterally located on mitochondrion-rich cells (MR Cell) of fish gill tissue, is responsible for hydrolyzing one ATP molecule (energy) to exchange three sodium ions for every two potassium ions (Evans et al., 1999). In doing so, an electrochemical gradient is produced that allows for the entrance of many other ions through apical channels, including calcium (Evans et al., 2005). As a result, Na^+/K^+-ATPase is especially important in controlling internal ionic balance. The Na^+/K^+-ATPase activity in a euryhaline species (*Anguilla Anguilla*) was shown to increase by a factor of 2.5 when moved from freshwater conditions to saltwater (Thomson and Sargent, 1977). Freshwater rainbow trout (*Oncorhynchus mykiss*) also showed an increase in Na^+/K^+-ATPase activity when exposed to increasing concentrations of seawater (Hawkings et al., 2004). Unlike carbonic anhydrase, these studies suggest that Na^+/K^+-ATPase activity increases in saltwater and freshwater species alike when exposed to increases in overall

salinity. When this activity is monitored during exposures to an increase in a single salt, specifically sodium bicarbonate, a decrease in activity results (Farag and Harper, 2014). This demonstrates that different dissolved ions may have varying roles in ionoregulation.

These results suggest that freshwater fish exposed to elevated dissolved ions may experience increased energy expenditure, either direct or indirect, with regards to carbonic anhydrase and Na^+/K^+-ATPase activity to combat osmoregulatory stress. This may leave less energy available for other important biological functions, such as growth and reproduction, ultimately leading to decreases in exposed fish populations.

Although extensive research has been done with regards to single salt exposures, particularly for sodium chloride and sodium bicarbonate, as well as changes in overall salinity, limited work has focused on the effects of sodium sulfate, or multi-ion exposures. Furthermore, most of the published literature has focused on either Na^+/K^+-ATPase or carbonic anhydrase enzymatic response to changes in salinity, but not both. Additionally, little work has been done to describe recovery of the activity of these enzymes following elevated dissolved ion exposures. This research, therefore, examined the response and recovery of both carbonic anhydrase and Na^+/K^+-ATPase in the gills of the fathead minnow during chronic exposures to sodium chloride, sodium sulfate, and sodium bicarbonate described as single anions, and in binary mixtures.

Materials and Methods

Pimephales promelas Culture Methods

Adult *Pimephales promelas* (fathead minnows) were reared in an in-house culture maintained at the Clemson Institute of Environmental Toxicology (CU-ENTOX, Pendleton, SC, USA). All fish were cultured and held in accordance with the appropriate

Institutional Animal Care and Use Committee protocols (Clemson University IACUC, AUP 2014-022 and AUP 2017-017). All fathead minnows were housed in a 3m by 1m water trough affixed to a ~ 350-gallon recirculating water system with biological filter. Culture water within the recirculating system was prepared using regent grade chemicals (96 mg/L NaHCO3, 60 mg/L CaSO4•H2O, 60 mg/L MgSO4, and 4.0 mg/L KCl) and Ultrapure (18 MΩ•cm resistivity) water (U.S. EPA, 2002). Temperature was recorded daily (°C), while alkalinity (mg/L $CaCO_3$), hardness (mg/L $CaCO_3$), pH, ammonia (ppt NH_3) and nitrate (ppt NO_2) were recorded weekly. Fish were fed Tetramin flake food purchased from Dr. Foster and Smith Aquatics, Inc., (Rhinelander, WI, USA).

Test Solutions

Four 300L carboys were used in order to prepare solutions 72-hours prior to test initiation to ensure all solutions were thoroughly and evenly dissolved. One carboy was used for each treatment: control, low, medium, and high. All solutions were created using the same reconstituted moderately hard water in which the fish were cultured, and the sodium-salts of chloride, bicarbonate, and sulfate. Sodium chloride (CAS 7647-14-5; Fisher Scientific, Atlanta, GA), sodium bicarbonate (CAS 144-55-8; Fisher Scientific, Atlanta, GA), and sodium sulfate (CAS 7757-82-6; Fisher Scientific, Atlanta, GA) were utilized in the preparation of test solutions. Once sodium-salts had been added to their respective carboys, an air stone was affixed to each to increase salt dissolution, as well as prevent the solution from becoming stagnant. Solutions were kept in the same temperature controlled room as test organisms to maintain the same temperature throughout the exposure. Samples were collected for every solution during water renewals and sent to the Clemson Agricultural Services Laboratory for ion quantification.

Bioassay Procedure and Experimental Design

Exactly 144 fathead minnows (between 0.75 and 2.5 g wet weight) were transferred from the recirculating culture system into a temperature controlled (22 ± 1°C) room, including a 16:8 light/dark cycle for each bioassay. The fathead minnows were then randomly divided into twelve 5-gallon aquaria containing 15L of reconstituted moderately hard water (U.S. EPA, 2002). Fish were then allowed to acclimate to the static nature of the bioassay 24-hours prior to test initiation once twelve fish were placed into each aquarium. At the start of each bioassay, aquariums containing twelve fish were divided into four treatment categories for both single-ion and binary mixture exposures. Single-ion exposures included control, low, medium, and high concentrations for each ion. Binary mixtures included control, low:high, medium:medium: and high:low concentrations, with the ion concentrations being the same as from the single-ion exposures. Each treatment was made up of three aquariums (n=3).

Once initiated, bioassays were continued for fourteen days. These fourteen days were split into a seven-day exposure period (days one through seven), and a seven-day post-exposure recovery period (days eight through fourteen). The first seven days, fish were exposed to either single ions or binary ion mixture solutions. Two sampling events, where one tank from each treatment was collected, occurred during this exposure period to understand the potential change in response over multiple days of exposure. The first sampling event occurred on day three of the exposure, while the final sampling event occurred on day seven for the exposure period. At the conclusion of the exposure period, fish from the final aquarium for each treatment were removed from the single ion

or binary ion mixture test solutions, and transferred into reconstituted moderately hard water. The final sampling event for the bioassay occurred on day fourteen to analyze post-exposure recovery.

During each sampling event, fish were removed from their aquaria and immediately euthanized in 1L moderately hard water mixed with 1.5g MS-222 buffered to pH = 7.5 with NaHCO3 (Clemson University Institute for Environmental Toxicology SOP 801-01-06). Fish were considered deceased 10-minutes after last operculum movement. The wet weight of each fish was recorded prior to dissection of gills, kidney and intestines. Each of the three tissues was placed on a separate piece of aluminum foil, folded and sealed, then flash frozen in liquid nitrogen and stored in a −80°C freezer for no longer than 3-months.

Test solution renewals occurred every 24-hours to ensure ammonia concentrations remain well below lethal limits (0.02 mg/L NH_3) for both exposures and post-exposure recovery periods. Water quality criteria including conductivity (µS/cm), pH, water hardness (mg/L $CaCO_3$), alkalinity (mg/L $CaCO_3$), temperature (°C), dissolved oxygen (mg/L), and ammonia (ppt NH_3) were performed. Alkalinity and hardness were only performed on the test solution prior to every 24-hour water renewal, and ammonia was only recorded for test solutions after the 24-hour exposure period. Fish were fed Tetramin flake food once daily. Tetramin was weighed prior to feedings at 200mg/aquarium to reduce any influence on enzymatic activity attributed to ions present in the feed. The last feeding occurred 24-hours prior to dissection to ensure the intestinal tract was mostly clear of all food substances.

Na^+/K^+-ATPase Activity Protocol

Methods used to quantify Na^+/K^+-ATPase activity followed those described by McCormick (1993) and Zahner (2009). These methods were developed to acquire samples through nonlethal gill sampling, and are adequate for use on small sample sizes. Because the tissues extracted from the adult fathead minnows were very small, the use of this method was ideal. Furthermore, it is one of the most common methods in use for this type of analysis, and is used throughout the published literature due to its reproducibility and high sensitivity (McCormick, 1993).

Gill tissues for each fish were removed from the −80°C freezer one treatment at a time (n=5), immediately removed from the aluminum foil pouch and placed into individual 2.0 mL micro-centrifuge tubes on ice. To each tube, 100 µL of SEI buffer and 25 µL of SEID buffer at approximately 4°C was added. To prepare the SEI buffer, 150 mM sucrose, 10 mM EDTA, and 5 0mM imidazole were added to a volumetric flask with Ultrapure (18 MΩ•cm resistivity) water until fully dissolved. Once dissolved, this mixture was transferred to a wide-mouthed flask. SEID buffer was prepared by adding 0.25 g sodium deoxycholate to 50 mL SEI buffer. Both solutions were placed in a 4°C refrigerator for 24-hours prior to use. Prior to use, the pH of both buffer solutions was adjusted to 7.5 with 0.1 M HCl. Next, the samples were thoroughly homogenized on ice using a hand-held IKA T-10 Basic Ultra-Turrax®. After homogenization, samples were centrifuged in an Eppendorf 5810R centrifuge at 8000*g* for 2-minutes at 4°C. Following centrifugation, 5 µL of supernatant were placed into four wells on a 96-well plate. To two wells, 150 µL of activation solution was added. The activation solution was

prepared by mixing 4-enzymatic units/mL of lactate dehydrogenase, 5-enzymatic units/mL of pyruvate kinase, 2.8 mM phosphoenolpyruvate, 3.5 mM adenosine triphosphatase (ATP), 0.22 mM nicotinamide adenine dinucleotide phosphate (NADH), and 50 mM imidazole. To the remaining two wells, 150 μL of deactivation solution was added. The deactivation solution was prepared by adding a known Na^+/K^+-ATPase specific inhibitor to an aliquot of the activation solution. Five inhibitors were tested in the present study to determine which, if any, were best suited for use in fathead minnows. The first inhibitor tested was ouabain since it iss the most studied and well-known Na^+/K^+-ATPase specific inhibitor. Both 1.5 mM and 13.0 mM ouabain were utilized, as well as 157 mM copper, 89.0 mM cadmium, 10 mM vanadate, and 6.5 mM digoxin. Both the activation solution and deactivation solutions were prepared less-than 24-hours before use, and pH adjusted to 7.5 with 0.1 M HCl. Just prior to analysis, 50 μL of a salt solution (189mM NaCl, 10.5mM MgCl2, 42mM KCl, and 50mM imidazole; pH adjusted with 0.1M HCl to pH = 7.5) was added to each well to initiate the reaction. Samples were then analyzed on a temperature controlled Molecular Devices Gemini microplate reader with UV detection at 340 nm every 45-seconds over 10-minutes. The resulting Na^+/K^+-ATPase activity was then calculated using the following equation:

(1) $$\frac{Average\ Vmax\ Activation - Average\ Vmax\ Deactivation}{Slope\ of\ the\ NADH\ Standard\ Curve} = Na+/K+ -ATPase\ Activity$$

Results were standardized to protein content, measured using a Protein Assay kit (Pierce, Rockford, IL, USA) and reported in mmol ADP/mg protein/hour.

Carbonic Anhydrase Activity Protocol

The Delta pH method is one of the most common methods used for measuring carbonic anhydrase activity today. Ease of use, utilizing relatively inexpensive

equipment, and providing accurate and reliable results are just some of the advantages of this technique (Henry, 1991). The Delta pH method employed for these studies follows the framework of Henry (1991); however, it includes modifications by Gervais and Tufts (1998) and Tufts et al. (1999). To start, gill samples were removed from –80°C storage and immediately transferred from their aluminum foil pouch to a 2 mL microcentrifuge tube containing an ice-cold TRIS buffer solution at a pH of 7.4 (10 mM TRIS base, 225 mM mannitol-D, and 75 mM sucrose; pH adjusted using 10% H_3PO_4). After homogenization with a hand-held IKA T-10 Basic Ultra-Turrax®, samples were centrifuged using an Eppendorf 5810R centrifuge at 8000*g* for 2-minutes at 4°C. Once centrifuged, either 2 µL or 5 µL (depending on the whole wet weight of each fish) of supernatant and 2 mL of TRIS buffers solution was added to a custom-made 2 mL glass well affixed to a recirculating water bath held at 4°C. The enzyme reaction was initiated with the addition of 40 µL of CO_2 saturated UltraPure (18 MΩ•cm resistivity) water, during which time the pH and mV change was recorded using a double, open junction micro-pH probe (Halo® HI13302, Hanna Instruments, Woonsocket, RI, USA) connected to the Hanna Lab App on an Apple® iPAD via Bluetooth Smart Technology. The catalyzed reaction for each sample was performed three times (n = 3). Another reaction to determine the uncatalyzed rate was also performed in duplicate (n = 2). This reaction was initiated in the same custom-made glass well with 2 mL TRIS buffer solution; however, the fish sample was omitted. The pH and mV change was recorded after the addition of 40 µL CO_2 saturated UltraPure (18 MΩ•cm resistivity) water. The uncatalyzed rate was indicative of the pH shift created solely by the CO_2 saturated UltraPure (18 MΩ•cm resistivity) water. Both the catalyzed and uncatalyzed reaction

rates for a 0.15 unit pH change were determined by plotting time (seconds) and mV. The average of both rates was divided by the buffer capacity to account for the rate of H^+ produced during the reaction. Buffer capacity was the change in mV, measured after the addition of 40 μL of 0.1N HCl to 2 mL of TRIS buffer solution in the same custom-made glass well, over time (seconds) divided by 4 μmoles. Carbonic anhydrase activity was calculated by subtracting the adjusted uncatalyzed reaction rate from the catalyzed reaction rate. Total carbonic anhydrase activity was then standardized to protein content, measured by a BCA Protein Assay kit (Pierce, Rockford, IL, USA) and reported in μmol H^+/mg protein/hour.

Statistical Analysis

All statistical analyses were performed utilizing JMP® Pro 12.0. For both Na^+/K^+-ATPase and carbonic anhydrase activities, five samples were analyzed. The average activities (n = 5) for each enzyme was determined and then plotted against treatment. Analysis of Variance (ANOVA) and Fisher's LSD was used to determine statistical differences between treatments and exposure days.

Results

Carbonic Anhydrase Activity

Chloride-only exposures to *P. promelas* resulted in no statistically significant changes in carbonic anhydrase activity on day 3 (*p* value: 0.3364) and day 7 (*p* value: 0.1091). A significant difference, however, occurred on Day 14, following the post-exposure recovery period. A significant increase in carbonic anhydrase activity occurred between the treatment containing low (8.56 mM) chloride and high (85.2 mM) chloride (*p* value: 0.0280*). The medium chloride treatment (34.2 mM chloride) did not result in

significant differences from the control (Figure 5.1; Figure 5.2; Table 5.1). No significant changes in carbonic anhydrase activity occurred between days or within treatments for sulfate-only exposures (Figure 5.3; Figure 5.4; Table 5.2; Table 5.8).

Exposures to bicarbonate-only further resulted in significant changes in carbonic anhydrase activity. By day 3, all treatments containing elevated bicarbonate resulted in a significant reduction in carbonic anhydrase activity from the control. However, by day 7, only the high treatment containing 23.8 mM bicarbonate remained significantly different (*p* value: 0.0049*). Following the 7-day post-exposure recovery period, all three treatments containing low (5.95 mM), medium (11.9 mM) and high (23.8 mM) bicarbonate resulted in statistically decreased carbonic anhydrase activity from the control. It was noted that a decrease in carbonic anhydrase activity occurred on day 7 for the control group, potentially resulting in less significant difference between treatments (Figure 5.5; Table 5.9). Significant changes in carbonic anhydrase did not result between day 3, day 7 and day 14 for 5.95 mM and 11.9 mM bicarbonate, whereas there was a significant reduction in activity on day 7 for the control group, and day 7 for the 23.8 mM bicarbonate treatment (Figure 5.6; Table 5.3).

Carbonic anhydrase activity significantly decreased from the control group on Day 3 of exposure to 8.35mM sulfate and 23.8mM bicarbonate (*p* value: <0.0001*), as well as 14.1 mM sulfate and 11.9mM bicarbonate (0.0172*). However, fish exposed to 35.2 mM sulfate and 5.95 mM bicarbonate did not result in any statistical difference from the control (*p* value: 0.1187) (Figure 5.8; Table 5.4). By day 7 and day 14, all treatments had similar carbonic anhydrase activities. Unlike sulfate with bicarbonate mixtures, chloride with bicarbonate mixtures did not induce a significant response until day 7,

when the treatment consisting of 34.2 mM chloride and 11.9 mM bicarbonate demonstrated a significant increase in carbonic anhydrase activity compared to the control (*p* value: 0.0044*). By day 14, both treatments containing 34.2 mM chloride:11.9 mM bicarbonate and 85.2 mM chloride: 5.95 mM bicarbonate demonstrated a statistically significant increase in carbonic anhydrase activity from the control (Figure 5.10; Table 5.5). Changes in carbonic anhydrase activity were only statistically significant between days in the treatment containing 85.2 mM chloride and 5.95 mM bicarbonate (Figure 5.9; Table 5.11). Mixtures containing 8.56 mM chloride with 35.2 mM sulfate, and 34.2 mM chloride with 14.1 mM sulfate were slightly decreased by exposure day 3; however, the 85.2 mM chloride with 8.35 mM sulfate was significantly reduced compared to the control (*p* value: 0.0144*). Furthermore, by exposure Day 7, 34.2 mM chloride with 14.1 mM sulfate was also reduced significantly compared to the control. Interestingly, day 14 indicated significantly reduced carbonic anhydrase activity in the treatment containing 8.56 mM chloride and 35.2 mM sulfate, which was not reduced at any other day during the exposure. The other two treatments (34.2 mM chloride with 14.1 mM sulfate and 85.2 mM chloride with 8.35 mM sulfate), which had previously been significantly reduced on days 3 and 7, were only slightly different from the control by day 14 (Figure 5.12; Table 5.6). The only treatment to produce a significant reduction in carbonic anhydrase activity over the 7-day exposure and 7-day post-exposure recovery period consisted of 8.56 mM chloride and 35.2mM sulfate. Although there was a slight increase in activity of the control group on day 7 of the exposure, it returned to similar activity by Day 14 (Figure 5.11; Table 5.12).

Preliminary Results: Na^+/K^+-ATPase Activity

Initially, methods for quantifying Na^+/K^+-ATPase activity were employed to demonstrate the enzymatic effects of sodium chloride, sodium sulfate, and sodium bicarbonate to *P. promelas*. Methods published by McCormick (1993) proposed the use of 0.5 mM ouabain, a known Na^+/K^+-ATPase specific inhibitor. However, it was noted by Zahner (2009) that *P. promelas* appeared to be fairly insensitive to ouabain exposure, and so 1.5 mM ouabain was utilized. Since the activation solution demonstrated all ATPase activity, and is not specific to any one of the ATPase enzymes, quantifying the difference between the total ATPase activity and residual ATPase activity after the addition of ouabain, the specific Na^+/K^+-ATPase activity can be calculated. However, there was no inhibition prompted by 1.5 mM ouabain, with the Vmax calculated as 46.0 μmol H+/minute for the activation solution, representing the total ATPase activity, and a Vmax of 40.1 μmol H+/minute for the deactivation solution containing 1.5 mM ouabain (Figure 5.13).

Due to the lack of inhibition, further preliminary studies utilizing higher concentrations of ouabain were also performed. Even at 13.0 mM oubain, the highest concentration tested due to the water solubility limit of ouabain, inhibition of Na^+/K^+-ATPase did not occur (activation Vmax: 27.1 μmol H+/minute; 13 mM ouabain Vmax: 26.4 μmol H+/minute) (Figure 5.14). To validate the accuracy of the procedure, copper, a known nonspecific ATPase inhibitor for *P. promelas*, was utilized. Unlike ouabain, copper did show a slight ATPase inhibition by decreasing the overall ATPase Vmax from 50 μmol H+/minute to 33.3 μmol H+/minute (Figure 5.15). Although copper is not

Na^+/K^+-ATPase specific, this inhibition experiment did show that inhibition was achievable and thus, the procedural methods were accurate.

The majority of the published literature utilizing ouabain demonstrates attainable Na^+/K^+-ATPase inhibition in mummichogs (*Fundulus heteroclitus*), Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (Morgan et al., 1997; Mancera and McCormick, 2000). To further demonstrate procedural accuracy, the same methods were employed using 1.5 mM ouabain on *F. heteroclitus* gill tissue. A significant reduction in Na^+/K^+-ATPase activity occurred between the activation solution (Vmax: 48.1 μmol H+/minute) and the deactivation solution (Vmax: 30.3 μmol H+/minute) (Figure 5.16). Because the methods were successful in inhibiting ATPase activity using copper in *P. promelas*, as well as ouabain-inhibition occurrence in a well-documented species, *F. heteroclitus*, the same procedure was used in all subsequent studies.

Ouabain, a member of the digitalis family, effectively inhibits potassium binding on the Na^+/K^+-ATPase enzyme. Although the effectiveness of ouabain has been demonstrated in many euryhaline species, including salmon and mummichogs, freshwater *P. promelas* were insensitive. Differences in these species must indicate that *P. promelas* has a slightly different structural conformity which limits the binding of ouabain. This structural difference may either result from environmental differences between species (freshwater versus euryhaline) or some evolutionary benefit.

Due to the insensitive nature of *P. promelas* to ouabain, other inhibitors were utilized to try and inhibit Na^+/K^+-ATPase in *P. promelas*, the first being *ortho*vanadate. This particular inhibitor is believed to mimic phosphate ions, and, as a result, inhibits phosphate binding on the enzyme during activation (Lindquist et al., 1973; Boyd and

Kustin, 1984). Although extreme inhibition occurred in the present study, it was not specific to Na^+/K^+-ATPase. As a result, all ATPase activity was impeded (Figure 5.17). Subsequently it was noted that vanadate is not specific to Na^+/K^+-ATPase alone, but instead, inhibits the activity of all ATPase enzymes (Boyd and Kustin, 1984). The final inhibitor tested was digoxin. Digoxin is similar to ouabain in that they are both members of the digitalis family, and specific for inhibiting Na^+/K^+-ATPase enzymes. However, little to no inhibition occurred (activation Vmax: 54.9 μmol H+/minute; digoxin Vmax: 48 μmol H+/minute) (Figure 5.18). A lack of inhibition for both ouabain and digoxin may indicate *P. promelas* is insensitive to all members of the digitalis group of inhibitors.

Due to the difficulty in obtaining a Na^+/K^+-ATPase specific inhibitor for *P. promelas*, final results were reported as total ATPase activity, measured by the activation solution. Although these results do not indicate changes in specific enzyme activities, they may indicate the allocation of more energy, in the form of ATP, for proper ionoregulatory processes.

Total ATPase Activity

Changes in total ATPase activity for *P. promelas* exposed to chloride-only did not show any significant changes on day 3 (*p* value: 0.3651) of exposure; however, by day 7 a significant difference between the four treatments occurred (*p* value: 0.0393*). Specifically, fish exposed to 34.2 mM and 85.2 mM chloride resulted in a significant increase in total ATPase activity from the control. By day 14, following a 7-day post-exposure recovery period, all treatments had similar activities (*p* value: 0.3783) (Figure 5.19; Table 5.13). The low treatment, containing 8.56 mM chloride, had a significant increase in total ATPase activity from day 3 to day 14 (*p* value: 0.0057*) and day 7 to

day 14 (*p* value: 0.0047*) indicating elevated activity post-exposure. However, it was also noted that an increase in total ATPase activity also occurred between day 3 and day 14 (*p* value: 0.0039*) and day 7 to day 14 (*p* value: 0.001*) for the control treatment (Table 5.19). Fish exposed to sulfate-only did not exhibit a significant change in total ATPase activity between days (Figure 5.22; Table 5.14) or within treatments (Figure 5.21; Table 5.20). Furthermore, results produced by bicarbonate-only exposures also revealed no significant differences in total ATPase activity between days (Figure 5.23; Table 5.15) or within treatments (Figure 5.24; Table 5.21).

Both mixtures containing bicarbonate did not produce any significant differences in total ATPase activity for either sulfate (Figure 5.25; Figure 5.26; Table 5.16; Table 5.22) or chloride (Figure 5.27; Figure 5.28; Table 5.17; Table 5.23).

Mixtures containing chloride and sulfate resulted in a statistically significant decrease in total ATPase activity across treatments for day 3, day 7 of exposure, and day 14, the post-exposure recovery period. By day 3, a significant reduction in overall ATPase activity occurred between the control group and mixtures containing 34.2 mM chloride and 14.1 mM sulfate, as well as 85.2 mM chloride with 8.35mM sulfate. However, the treatment containing 8.56 mM chloride and 35.2 mM sulfate was not significantly different from the control group by day 3. By day 7 and day 14, both treatments continued to show a significant reduction in total ATPase activity (Figure 5.30; Table 5.18). There were no significant changes in total ATPase activity between exposure days for each treatment (Figure 5.29; Table 5.24), indicating that once reduced ATPase activity occurred, it did not improve following a post-exposure recovery period.

Discussion

Aquatic systems are naturally comprised of many different dissolved ions, which are reflective of their surrounding environment. The concentration of dissolved ions in these aquatic systems ultimately controls the salinity of each system. A low salinity environment, such as a freshwater system, consists of very low dissolved ion concentrations. Because these dissolved ions are critical for various functions within living organisms, freshwater fish have adapted to the osmotic pressures characteristic of their surrounding environment. One such adaptation includes the use of active transport in order to move ions against their concentration gradient, from the low ion concentration of their external environment, to the high ion concentration typical of their internal environment (refer to Figure 1.1; Evans, 1980; Evans et al., 1999; Perry et al., 2003; Evans et al., 2005; Gilmour and Perry, 2009). Because freshwater organisms are adapted to managing low dissolved ion concentrations, it is unclear exactly how an increase in these ions would impact active transport.

Elevated dissolved ions are of concern for freshwater organisms mainly due to the many anthropogenic activities that lead to increased dissolved ions, as well as changes in ionic composition, of their surrounding environment (Timpano et al., 2010; Pond et al., 2004; Farag and Harper, 2014*a*; Brittingham et al., 2014). Previous studies have reported adverse effects ranging from changes in ecosystem structure, reproductive impairment, decreased growth and even mortality for freshwater organisms exposed to increased ion concentrations (Dickerson et al., 1997; Mount et al., 1997; Tietge et al., 1997; Blasius and Merritt, 2002; Kennedy et al., 2003; Soucek and Kennedy, 2005; Vosyliene et al., 2006; Pond et al., 2008; Soucek et al, 2011; Farag and Harper, 2014*b*; Mount et al., 2016;

Wang et al., 2016; Johnson-Couch (Chapter 2); Johnson-Couch (Chapter 3)). It has been previously suggested that an increase in energy used for ionoregulation and active transport may decrease the energy available for other critical functions, hence a decrease in growth and reproduction (Farag and Harper, 2014*a*). However, a clear toxicity gradient is exhibited, where some ions produce a more toxic response than others. In ion-only studies, the chronic toxicity of divalent ions (SO_4^{2-}, Mg^{2+}, Ca^{2+}) elicited a more pronounced response in reproduction of *Ceriodaphnia dubia* and growth of *Pimephales promelas* than their monovalent counterparts (Na^+, Cl^-, HCO_3^-) (Johnson-Couch (Chapter 2); Johnson-Couch (Chapter 3)). Although this finding is interesting, increases in dissolved ions in the environment do not occur as one constituent, but in mixtures. How these multi-ion mixtures interact at the site of action, predominantly the gill could greatly influence the energy necessary for managing excess dissolved ions.

Mitochrondrion-Rich (MR) cells and pavement cells, located on the gill of freshwater organisms, are responsible for regulating the movement of ions between their external and internal environment (refer to Figure 1.1). It is well known that sodium channels located on the apical membrane, as well as Na^+/K^+-ATPase enzymes on the basolateral membrane of these MR cells are responsible for combating the diffusive loss of sodium through paracellular junctions on the apical membrane (Evans et al., 2005). Although the sodium channel is responsible for the transport of sodium into the MR Cell cytoplasm, Na^+/K^+-ATPase is principally responsible for sodium gaining access to the plasma, and ultimately the internal environment of the organism (Evans et al., 1999. Furthermore, H^+ exits the MR cell through an H^+-ATPase located on the apical membrane, which drives the movement of sodium into the MR cell cytoplasm (Evans et

al., 1999). Not only are these series of transporters, channels, and exchangers important for maintaining ionic regulation, but also acid-base balance (Perry et al., 2003; Evans et al., 2005; Marshall and Grosell, 2005; Gilmour and Perry, 2009). The acid-base balance capabilities of fish is predominantly controlled by carbonic anhydrase, which is located within the cytoplasm of MR cells. Endogenous CO_2 waste diffuses into the MR cell cytoplasm, where carbonic anhydrase rapidly converts it with water into HCO_3^- and H^+ ions. The H^+ ion is then excreted through the H^+-ATPase, while allowing the entrance of one Na^+ ion through the sodium channel. Additionally, one HCO_3^- is excreted while one Cl^- ion enters through the Cl^-/HCO_3^- exchanger. Changes in external ion concentrations, which have been shown to negatively impact freshwater organisms, could be driven by an effect on ionoregulatory systems. Due to the large interplay between these ionoregulatory enzymes, an effect on one, could lead to an effect on another or all, resulting in the negative impacts on growth and reproduction.

Because Na^+/K^+-ATPase and carbonic anhydrase prompt most ionoregulatory enzymatic actions, as well as utilize energy either directly or indirectly to perform their functions, the goal of the present study was to understand the changes in their activity caused by elevated dissolved ions, both as single ions and multi-ion mixtures. Any changes could reflect on ionoregulatory impairment, and/or energy allocation. Unfortunately, due to difficulties in identifying a Na^+/K^+-ATPase specific inhibitor in *P. promelas*, only total ATPase activity could be reported. Although this limits the interpretation of the results, changes in total ATPase activity could still provide insights into any ionoregulatory disturbances and changes in energy allocation.

Three-day exposures to sodium chloride did not produce any significant changes in both carbonic anhydrase and total ATPase activity, indicating that freshwater fish can withstand concentrations as high as 85 mM chloride without any obvious initial consequence. By day 7, however, fish exposed to 34.2 mM and 85.2 mM chloride showed significantly elevated ATPase activity, increasing by roughly 42%, while carbonic anhydrase activity remained stable. The estimated Effective Concentration (EC) reducing larval *P. promelas* growth by 50% compared to the control was 50.7 mM chloride (Johnson-Couch (Chapter 3). This corresponds approximately with the chloride concentrations producing elevated ATPase activity, which could indicate a reduction in growth due to the allocation of more energy for ionoregulatory purposes. Although it is quite well recognized that an apical Cl^-/HCO_3^- exchange protien is responsible for Cl^- uptake by MR cells (Maetz and Garcia-Romeu, 1964; Maetaz, 1976; Perry, 1997), it is still unclear what truly drives its function. The possibility of a V-type proton pump located on the basolateral membrane of the MR cell may provide the electrochemical gradient necessary for Cl^-/HCO_3^- exchange to occur (Piermarini and Evans, 2001). During this exchange, one chloride ion enters into the cytoplasm of the MR cells, while one bicarbonate ion exits. For chloride in the external media to enter through the gill tissue, more bicarbonate must be present inside the cyptoplasm of the MR cell for an exchange to occur. In fact, an increase in CO_2 efflux has been demonstrated in goldfish (*Carassius auratus*) during exposure to high external chloride concentrations (Dejours, 1969). This may indicate that upon exposures to high external chloride concentrations, freshwater fish increase chloride influx and bicarbonate efflux.

If more bicarbonate is present within the fish, measured as CO_2 excretion, it would be expected that an upregulation of carbonic anhydrase would result. However, when sodium chloride increased in concentration over the 7-day exposure period, no significant changes in carbonic anhydrase occurred. It has been suggested that a sodium and chloride co-transporter on the apical membrane of the MR Cell is present, and could potentially lead to an overall increase in total ATPase activity (Evans, 2011). The presence of a sodium and chloride co-transporter would ultimately bypass the Cl^-/HCO_3^- exchange system and thus, not affect carbonic anhydrase activity.

Although it is believed that chloride and bicarbonate are dependent on one another for translocation, in the present study, bicarbonate-only exposures did not significantly affect total ATPase activity similar to chloride-only exposures. Instead, bicarbonate-only exposures resulted in significant decreases in carbonic anhydrase activity, by approximately 64%, as early as day 3 of the exposure. By day 7, 5.95 mM and 11.9 mM bicarbonate were not significantly different from the control; however, 23.8 mM bicarbonate remained significantly lower. It is important to note that on day 7, the control group had a significant reduction in carbonic anhydrase activity itself, and may be the reason why some differences were not significant. However, because the highest treatment (23.8 mM bicarbonate) was still significantly lower, it speaks volumes to the hindrance of carbonic anhydrase activity by bicarbonate, or more specifically, sodium bicarbonate. The reason for a decrease in carbonic anhydrase activity in the control fish on day 7 is unknown. Although it may be due to stress in the organism, it is more likely variation within each treatment. Large 95% confidence intervals surrounding the control group, especially on days 3 and day 7 of the post-exposure recovery period (day 14)

could indicate large typical differences that some fish display in ionoregulation. Changes in sodium plasma concentrations are known to fluctuate within freshwater fish, depending on where the fish is in its osmotic cycle (Prior et al., 1995). Random sampling may contribute to the selection of fish at different points, whether low or high, in their osmotic cycle. Nevertheless, a decrease in control carbonic anhydrase activity was short-lived, as the control treatment carbonic anhydrase rebounded following the 7-day post-exposure recovery period, and was not significantly different from day 3 activity. Additionally, all treatments following the 7-day post-exposure recovery period again showed significant decreases in carbonic anhydrase activity, further demonstrating that an anomaly within the control fish on day 7 was most plausible.

The movement of bicarbonate from the plasma of freshwater fish, in exchange for chloride, is most likely related to the need for maintaining critical blood pH. It may also be related to the movement of bicarbonate from an area of high concentration within the fish, to an area of low concentration in the external environment. However, when the bicarbonate concentration in the external environment exceeds what is physiologically tolerable for freshwater fish, they may no longer be able to excrete bicarbonate. A buildup of bicarbonate within MR cells may alter the internal pH, ultimately reducing further bicarbonate production and carbonic anhydrase activity. An accumulation of bicarbonate within the plasma of fish has been demonstrated in rainbow trout (*Oncorhynchus mykiss*) following exposure to alkaline waters (pH = 10.5) (McGeer and Eddy, 1998). Without proper function of carbonic anhydrase enzymes, CO_2 may accumulate within the fish, leading to acidosis. Previous studies have shown that freshwater fish quickly respond to acidotic conditions by decreasing Cl^-/HCO_3^- exchange,

leading to a decrease in both chloride uptake and bicarbonate efflux (Goss et al., 1992). In addition, the influx rate of sodium has been shown to increase drastically during times of acidosis, which should correspond to increases in ATPase activity (Cameron, 1976). However, changes in total ATPase activity did not occur during bicarbonate-only exposures. This is unlike previous studies that demonstrate a decrease in Na^+/K^+-ATPase enzyme amount and activity in *P. promelas* following chronic sodium bicarbonate exposures (Farag and Harper, 2014*b*). However, this decrease does seem to be time dependent. Farag and Harper (2014*b*) found significant decreases in Na^+/K^+-ATPase at 300 mg/L $NaHCO^3$ at day 60, but not day 37. This could indicate that seven-day exposures are not long enough to elicit a response in ATPase activity. Additionally, chloride is actively pumped out of the gill by the indirect energetic coupling with a basolateral V-type ATPase activity in saltwater fish (Maetz, 1972). Although this occurrence hasn't been explicitly documented in freshwater fish, it has been suggested that chloride uptake does require an active transport system (Kerstetter and Kirschner, 1972). If this is true, freshwater fish may utilize some form of active transport to translocate chloride and bicarbonate. Changes in bicarbonate efflux, as well as carbonic anhydrase activity, may be responsible for overall decreases in Na^+/K^+-ATPase activity over time if activity of this V-type ATPase is in fact coupled to Cl^-/HCO_3^- exchange. In fact, during inhibition of carbonic anhydrase, a complete inhibition of chloride uptake and a 75% reduction in sodium uptake has been exhibited in goldfish (Maetz and Garcia Romeu, 1964). Because of this limited uptake in physiologically important ions, toxicity of bicarbonate may be linked to a chloride or sodium deficiency.

Precipitation of calcium carbonate, a white solid, on the test chambers during elevated bicarbonate exposures has also been linked to increases in bicarbonate concentration within fish (Farag and Harper, 2014*b*). As the bicarbonate concentration rises, so does the rate of precipitation of calcium carbonate, ultimately reducing the concentration of water-soluble calcium in the water column. Among its many functions, calcium is known to reduce cell permeability in freshwater fish gills, particularly with regards to sodium (Hunn, 1985). If the water-soluble calcium concentration is reduced due to elevated bicarbonate concentrations, it could subject the gill to a higher influx of sodium through unprotected paracellular junctions.

One issue with evaluating total ATPase activity, and not the changes in specific ATPases, is the fact that certain ATPases may be upregulated while others are downregulated so that the overall total ATPase level remains unchanged. For example, while it has been reported that Na^+/K^+-ATPase activity decreased during elevated bicarbonate exposures, it has been suggested that Ca^{2+}-ATPase upregulates in order to combat low calcium concentrations in external media (Farag and Harper, 2014*b*; Hunn, 1985; Marshall, 2002). Nevertheless, regardless of the differences in specific ATPase activity, increased energy allocation in the gill through ATPases and carbonic anhydrase for ionoregulatory purposes does not seem to be the primary route of bicarbonate toxicity with respect to decreased growth in *P. promelas*.

Since the gill is impermeable to divalent anions, such as SO_4^{2-}, it is not surprising that an association between sulfate-only exposures and changes in gill total ATPase and carbonic anhydrase activity was not found (Garcia-Romeu and Maetz, 1964; Maetz, 1971). Changes in sodium influx rates, however, have been reported in goldfish

(*Carassius auratus*) following sodium sulfate exposures (Garcia-Romeu and Maetz, 1964). It would be expected that that an increase in sodium influx would result in higher ATPase activity, primarily attributable to Na^+/K^+-ATPase. Furthermore, sodium concentrations utilized in the present study were similar to the previously estimated sodium EC_{50} for growth effects in *P. promelas* (73.9 mM) (Johnson-Couch (Chapter 3)). If decreases in growth occurred due to the reallocation of energy for ionoregulatory purposes, an increase in active transport systems would have been expected, however, changes in total ATPase and carbonic anhydrase activity within the gill of *P. promelas* did not occur in the present study. Taking these aspects into consideration, perhaps the main contributor to toxicity is sulfate controlled. This has also been suggested by previous studies following acute and chronic sodium sulfate exposures to multiple species (Mount et al., 1997; Soucek et al., 2011; Johnson-Couch (Chapter 3)).

Due to the impermeability of the gill to sulfate, this particular ion must have a mechanism of action separate from the gill. The most likely route of toxicity is the intestinal tract. Although saltwater fish drink copious amounts of water to combat osmotic water loss, freshwater fish typically drink much less due to passive water gain (Maetz, 1971). However, it would be expected that freshwater fish would ingest larger quantities of water if the ion concentration in the external environment exceeded that present inside the fish. In situations where sodium and sulfate are present at elevated concentrations, along with increased water uptake, the fish would also ingest high concentrations of sodium and sulfate. Sulfate, and other divalent ions such as magnesium and calcium are known to increase monovalent ion (sodium and chloride) uptake with the gastrointestinal tract of mammals, and may be the case in fish as well (Ingraham and

Visscher, 1936; Ingraham and Visscher, 1938; Shehadeh and Gordon, 1969). Additionally, euryhaline fish excrete more sulfate when exposed to elevated magnesium sulfate, suggesting they do not utilize much energy in the active uptake of sulfate along the gastrointestinal tract (Oikari and Rankin, 1985). If this is the case, perhaps increased energy allocation for ionoregulation is occurring within the gastrointestinal tract and not necessarily the gills. This would suggest that in fact, sodium is having a toxic effect on freshwater fish, most likely due to changes in electrochemical gradients, but is enhanced through elevated sulfate concentrations.

Changes in gill total ATPase activity did not occur in mixtures containing sulfate and bicarbonate, which was to be expected since both ions independently resulted in no significant changes in total ATPase activity. However, a significant reduction in carbonic anhydrase was apparent on day 3 in all treatments, with fish returning back to normal by day 7. This suggests that bicarbonate elicited an initial response within the gill of *P. promelas*, but due to the presence of sulfate, the fish were able to recover. The initial toxicity from higher bicarbonate concentrations may be the result of decreased chloride uptake along the gills. Recovery by exposure day 7 may be due to the increased absorption of chloride along the gastrointestinal tract by elevated sulfate concentrations. Alternatively, chloride and bicarbonate mixtures resulted in significantly elevated carbonic anhydrase activities by treatments consisting of higher chloride and lower bicarbonate concentrations by exposure day 7, while a decrease in total ATPase activity occurred on day 3 in treatments consisting of lower chloride and higher bicarbonate concentrations. These results are unlike bicarbonate-only and chloride-only results, where bicarbonate-only produced a decrease in carbonic anhydrase activity with no effect

on total ATPase, and chloride-only resulted in an increase in total ATPase activity, with no effect on carbonic anhydrase. These results are further evidence of the complex nature of dissolved ion toxicity.

Growth effects exhibited by *P. promelas* following exposure to mixtures containing chloride and bicarbonate resulted in an additive interaction, meaning that the addition of one ion does not affect the toxicity of the second. Although it does not appear enzymatic activity necessarily acts in an additive manner, it does seem as though the addition of bicarbonate prevents chloride effects on ATPase activity. Also, the addition of chloride prevents bicarbonate effects on carbonic anhydrase activity.

Interestingly, combinations of chloride and sulfate significantly reduced total ATPase activity in treatments containing 34.2 mM chloride and 14.1mM sulfate, as well as 85.2 mM chloride and 8.25 mM sulfate. There was no response of total ATPase activity when chloride was low (8.96 mM) and sulfate was high (35.2 mM). This makes sense considering all sulfate-only treatments and the low (8.96 mM) chloride treatment during chloride-only exposures did not have any significant effects on total ATPase activity. However, it is interesting that combinations with higher chloride concentrations resulted in such a drastic decrease on gill total ATPase activity. Perhaps the decrease in total ATPase activity is not necessarily due to cellular destruction or improper enzyme function. If freshwater fish drink large volumes of water to combat osmotic water loss during periods of elevated dissolved ions, they would also ingest high quantities of sodium, sulfate and chloride. The elevated sulfate would lead to increased absorption of sodium and chloride along the gastrointestinal tract, thus reducing the activity needed to increase uptake along the gill. Furthermore, if freshwater fish reach a maximum tolerable

sodium and chloride concentration, they may reduce activity uptake along the kidney and produce highly concentrated urine. This could also explain why less-than-additive effects exhibited by growth in *P. promelas* occurred. If the organism requires less energy for ionoregulation along the gill and kidney, they may be able to utilize more energy for feeding, escaping predation, reproduction, and other important biological functions.

Due to the possibility for changes within the other ionoregulatory important tissues, such as intestines and kidney, in *P. promelas* following certain exposures, it may be beneficial to measure total ATPase and carbonic anhydrase activity within these tissues as well. This could aid in the understanding of toxicity for these specific ions. Moreover, specific ion concentrations within the plasma of fish could further explain if ion translocation is actually occurring via passive diffusion, specifically for ions that did not seem to result in any change in enzymatic activity.

It may also be important to note that although most fish species utilize Cl^-/HCO_3^- exchangers, that may require energy through basolateral ATPases, others passively transport chloride without the use of ATP (Marshall and Grosell, 2005). For example, mummichogs (*Fundulus heteroclitus*) utilize active transport for sodium uptake, but not chloride (Wood and Marshall, 1994; Patrick et al., 1997; Tomasso and Grosell, 2005; Bucking et al., 2013). *F. heteroclitus* is also unique in that it lacks a stomach (Babkin and Bowie, 1928; Bucking et al., 2013). This could be of importance considering *P. promelas* is a member of the *Cyprinidae* family, which also consists of carps, a group of fish that also lack true stomachs (Kraatz, 1924). Specifically, *P. promelas* lack gastric glands, a structure which aids in acid secretion. This means that *P. promelas* does not utilize acid to break down food particles, and instead maintains a pH similar to their

external environment in the digestive tract (Day et al., 2011). This similarity between *P. promelas* and *F. heteroclitus* could indicate that they also osmoregulate similarly, particularly with regards to intestinal uptake. It would be interesting to assess the actual processes employed during ionoregulation in *P. promelas* to ensure utilizing carbonic anhydrase activity as a means to associate energy usage and chloride uptake is adequate.

Similarities between concentration-response curves of different contaminants have been suggested to have similar modes-of-action (van der Geest et al., 2000). Chronic exposures estimating growth effects due to elevated ion exposures resulted in similar concentration-response curves for sodium, bicarbonate, and chloride, while sulfate was different. This could mean that sulfate, unlike sodium, bicarbonate, and chloride, does not actually affect ion uptake along the gill, but instead increases uptake of sodium and chloride within the gastrointestinal tract of freshwater fish contributing to its toxic effect. Differences in enzymatic activities and toxicities occurred between chloride, bicarbonate and sulfate ions and ion combination exposures, indicating that these particular ions elicit different responses in the gill of *P. promelas*. However, the belief that freshwater fish utilize more energy in order to ionoregulate and maintain proper internal ion concentration during times of elevated ion exposure may still hold true, although the energetic cost for each ion, and ion combination may be different.

Conclusions

Freshwater organisms have been suggested to alter energy allocation for proper ionoregulatory function and maintaining proper ion balance when exposed to elevated dissolved ions at concentrations exhibiting sub-lethal effects. By doing so, freshwater organisms would decrease the energy available for survival and biological success. In the

present study, total ATPase and carbonic anhydrase activity were measured in order to elucidate a mechanism of action for elevated dissolved ions. The total ATPase and carbonic anhydrase activity of adult *P. promelas* gill tissue was different between organisms exposed to elevated sulfate, chloride, bicarbonate and sodium, as single ions and in multi-ion mixtures. Most effects on carbonic anhydrase activity were in response to elevated bicarbonate, while total ATPase was mostly affected by sodium and chloride. Sulfate did not produce a response in either total ATPase or carbonic anhydrase activity of gill tissue. Because the gill is impermeable to divalent anions, such as sulfate, it may be that sulfate increased the uptake rate of sodium along the gastrointestinal tract. As a result, further studies should be completed on other ionoregulatory important tissues, including kidney and intestines, in order to understand the toxicity of elevated ions throughout the entirety of the organism. Measuring ion concentrations within the plasma of each organism would further our understanding of active uptake versus passive diffusion of freshwater organisms exposed to higher salinity solutions. Differences between enzymatic activities in the gill tissue of *P. promelas* exposed to elevated dissolved ions indicate that each ion and ion combination exerts its own energetic cost by either increasing or decreasing enzymatic activity.

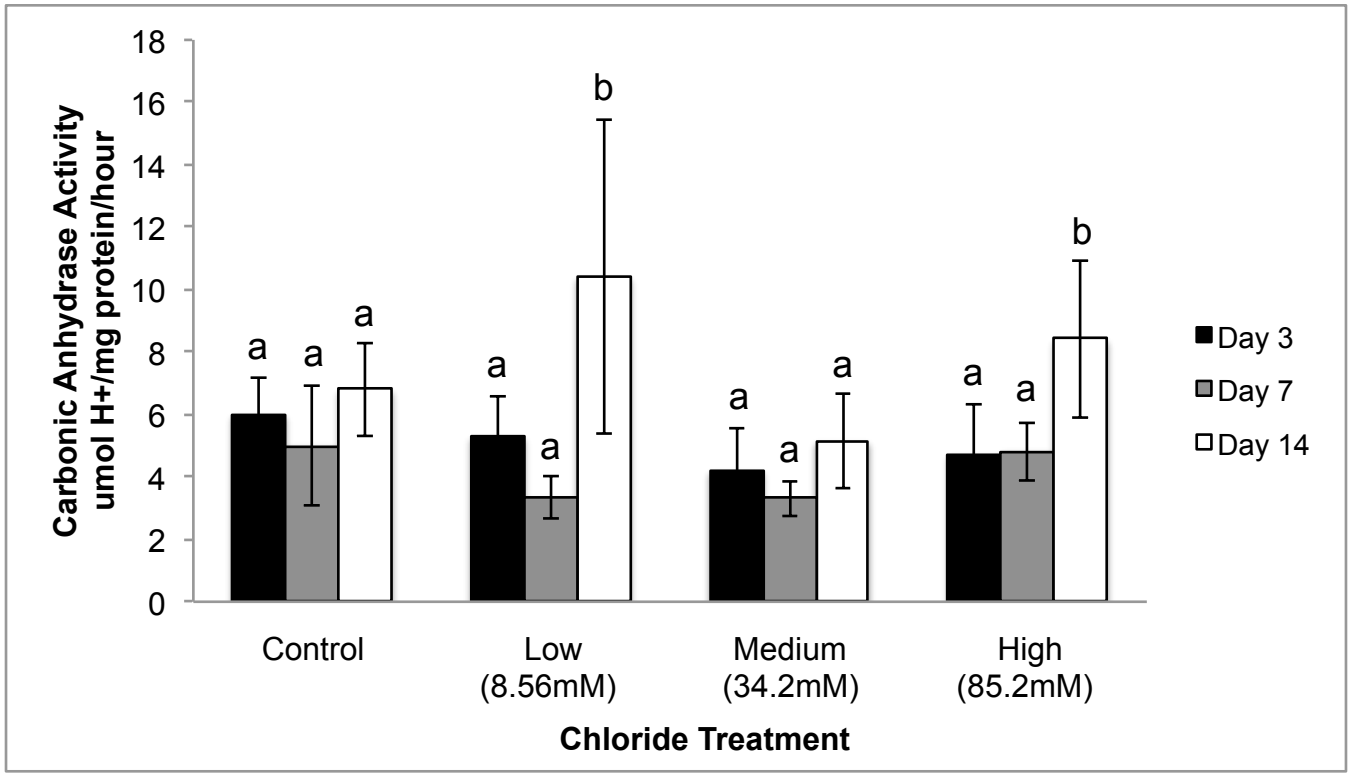

Figure 5.1. Carbonic anhydrase activity in *P. promelas* gill tissue following chloride-only exposures. Bars represent average carbonic anhydrase activity measured as μmol H^+/mg protein/hour ± 95% confidence intervals ($\alpha = 0.05$). Significant differences within treatments are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

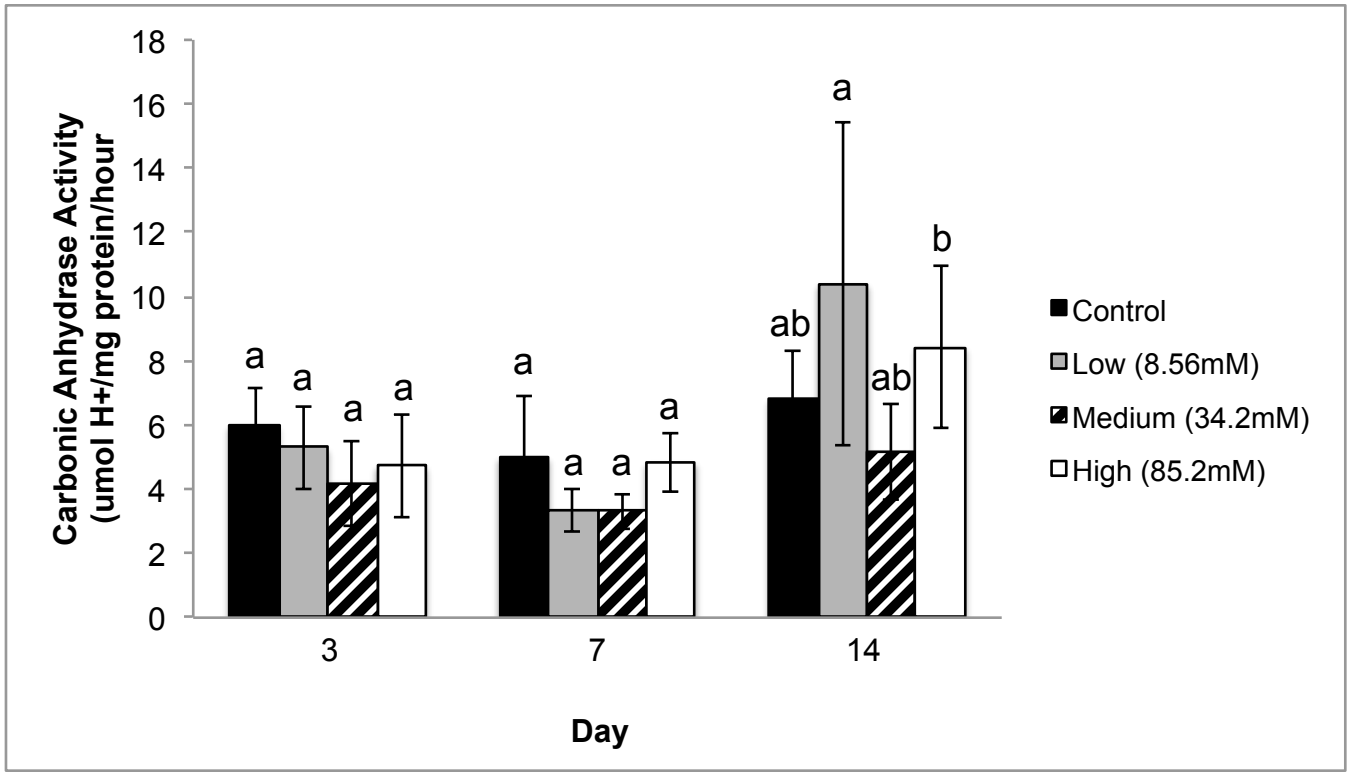

Figure 5.2. Changes in carbonic anhydrase activity in *P. promelas* gill tissue following chloride-only exposures. Bars represent average carbonic anhydrase activity measured as μmol H^+/mg protein/hour ± 95% confidence intervals ($\alpha = 0.05$). Significant differences within sampling days are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

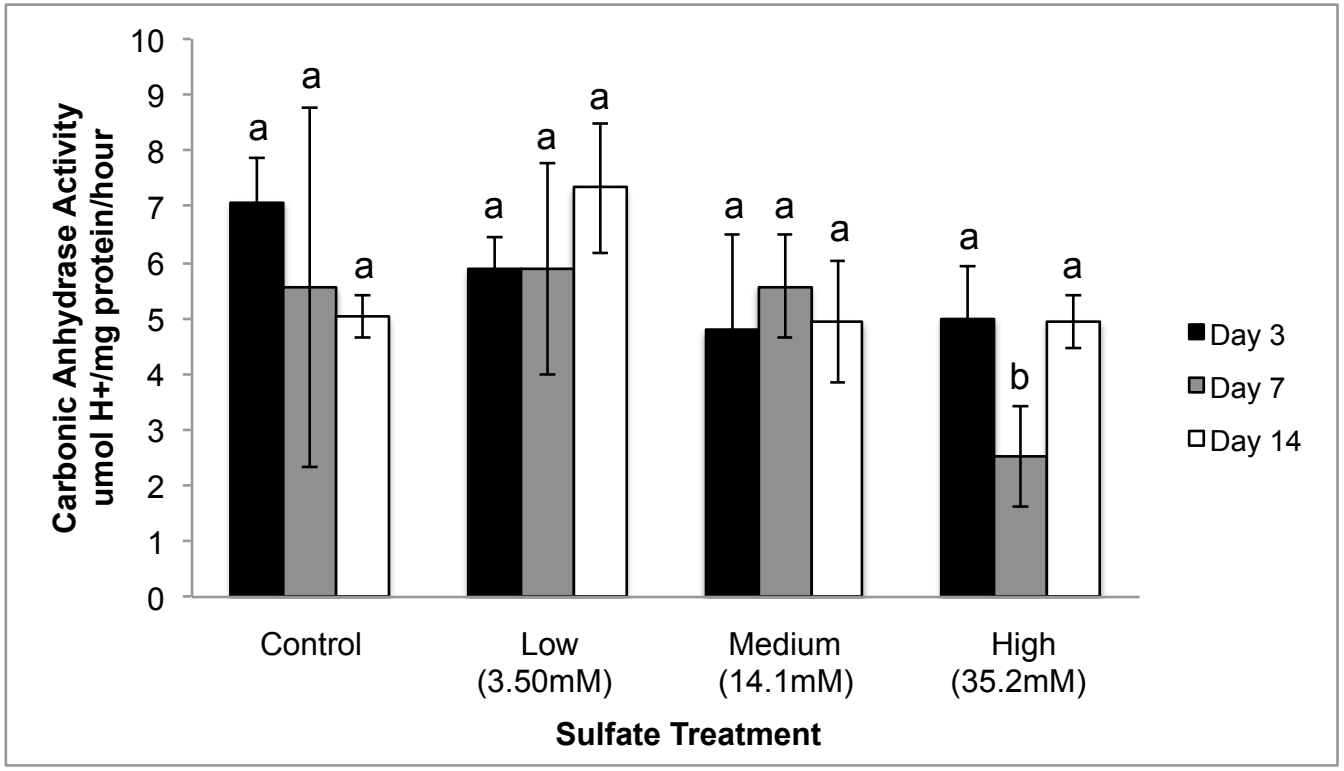

Figure 5.3. Carbonic anhydrase activity in *P. promelas* gill tissue following sulfate-only exposures. Bars represent average carbonic anhydrase activity measured as μmol H^+/mg protein/hour ± 95% confidence intervals ($\alpha = 0.05$). Significant differences within treatments are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

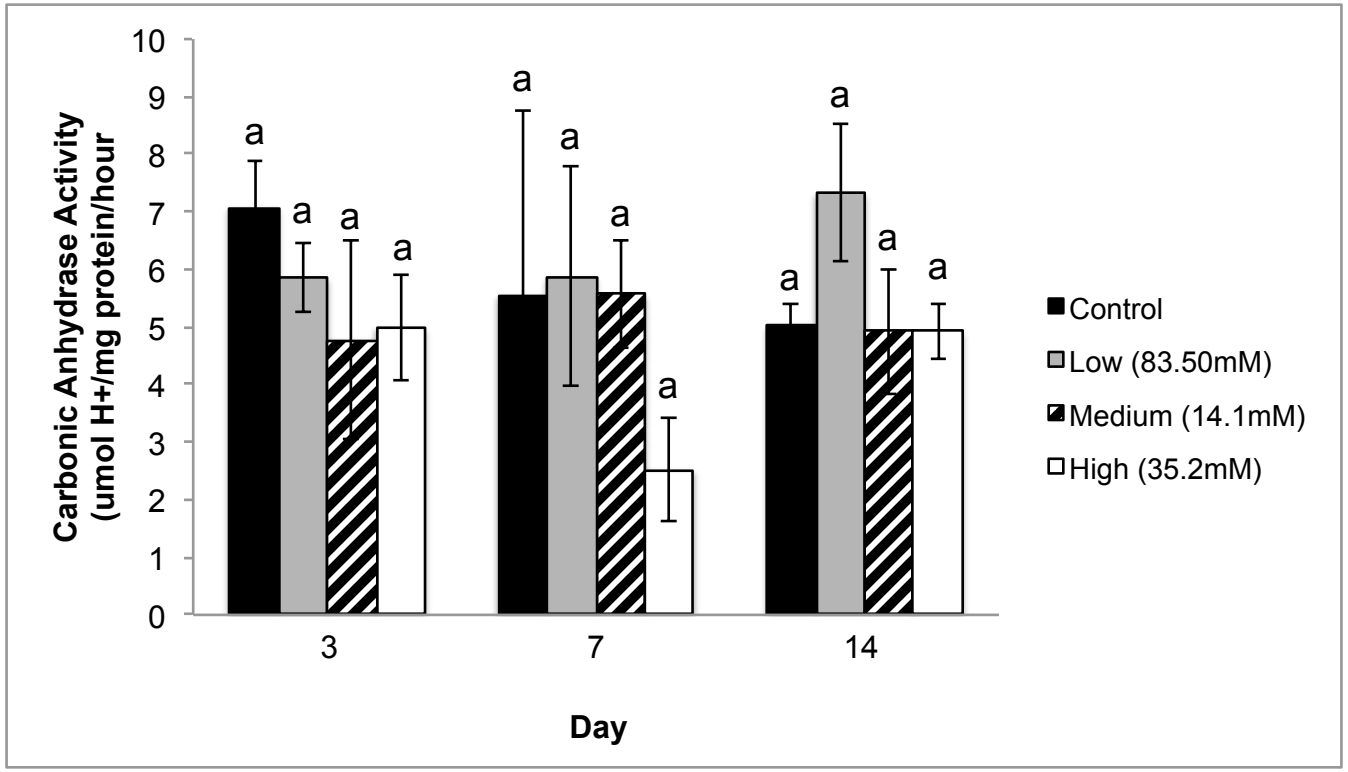

Figure 5.4. Changes in carbonic anhydrase activity in *P. promelas* gill tissue following sulfate-only exposures. Bars represent average carbonic anhydrase activity measured as μmol H^+/mg protein/hour ± 95% confidence intervals ($\alpha = 0.05$). Significant differences within sampling days are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

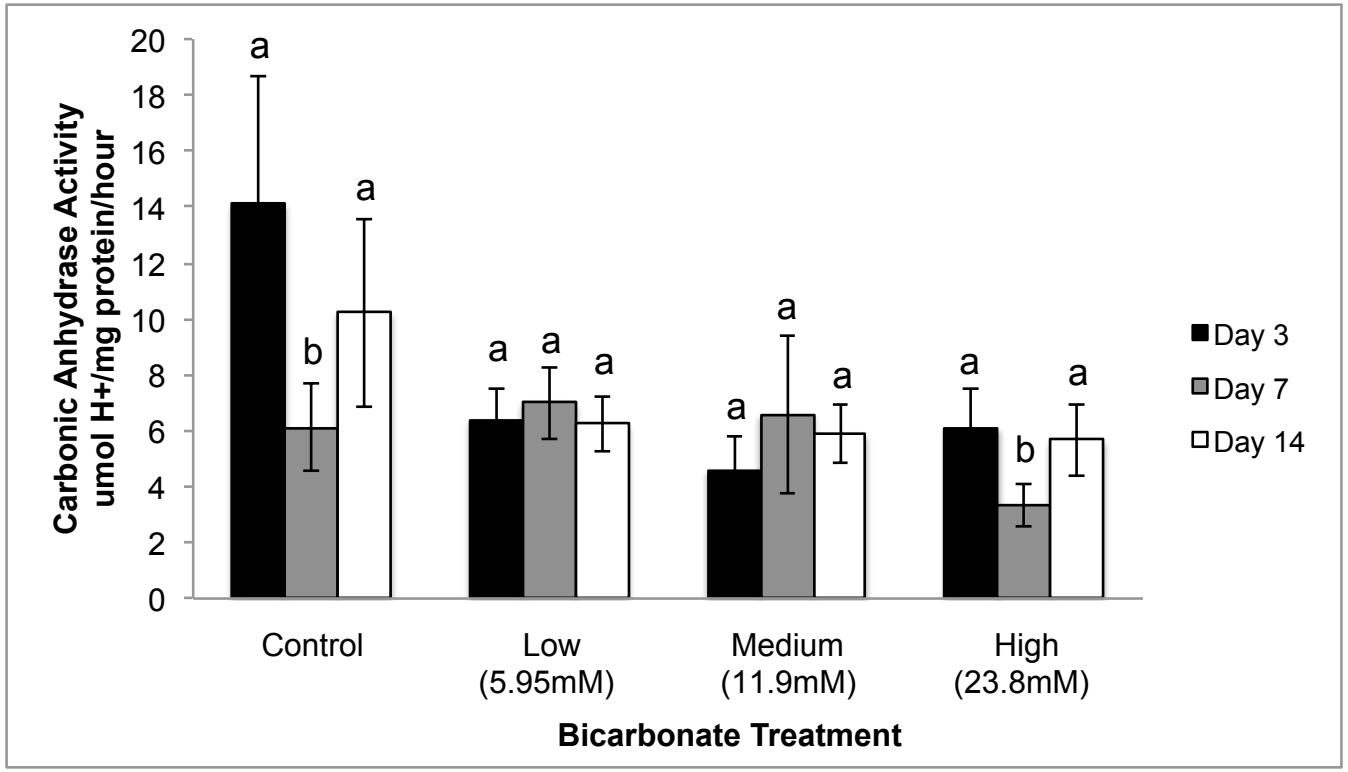

Figure 5.5. Carbonic anhydrase activity in *P. promelas* gill tissue following bicarbonate-only exposures. Bars represent average carbonic anhydrase activity measured as μmol H^+/mg protein/hour ± 95% confidence intervals ($\alpha = 0.05$). Significant differences within treatments are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

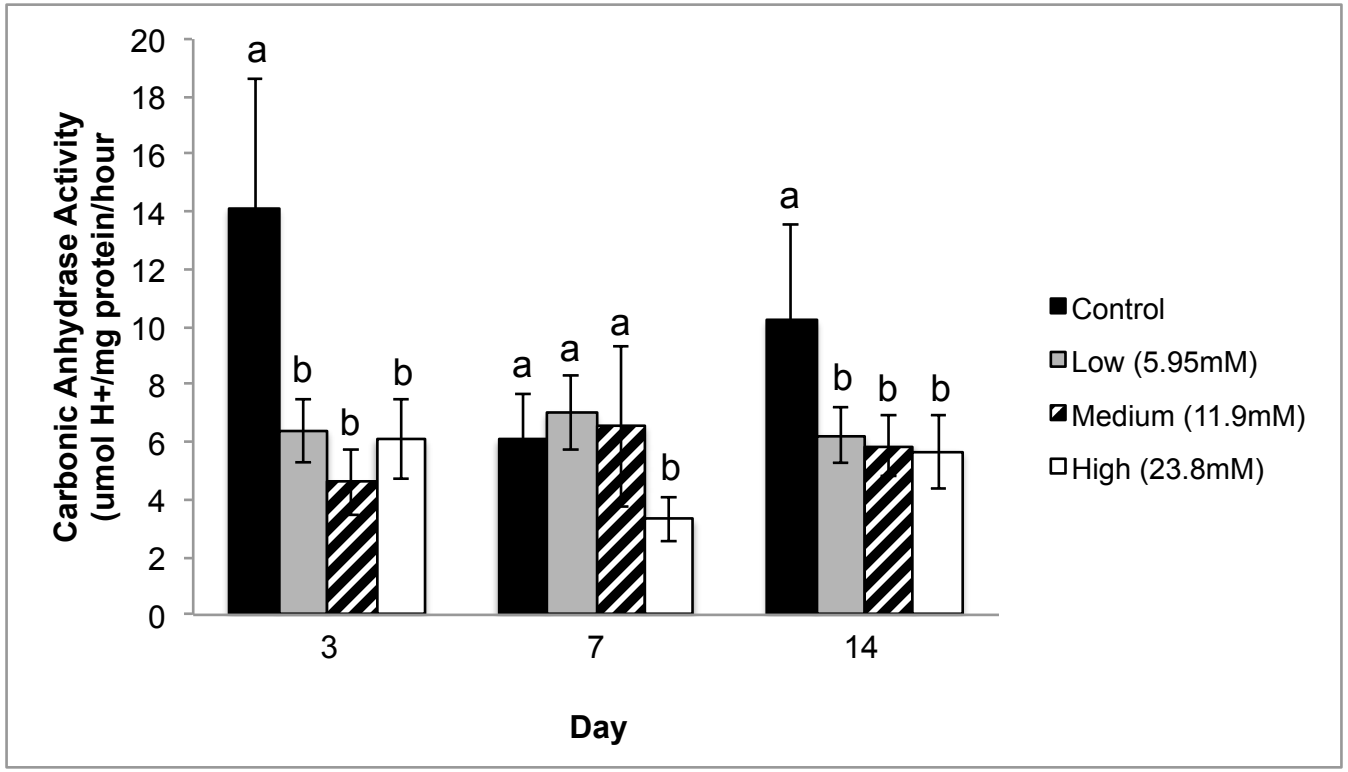

Figure 5.6. Changes in carbonic anhydrase activity in *P. promelas* gill tissue following bicarbonate-only exposures. Bars represent average carbonic anhydrase activity measured as μmol H^+/mg protein/hour ± 95% confidence intervals ($\alpha = 0.05$). Significant differences within sampling days are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

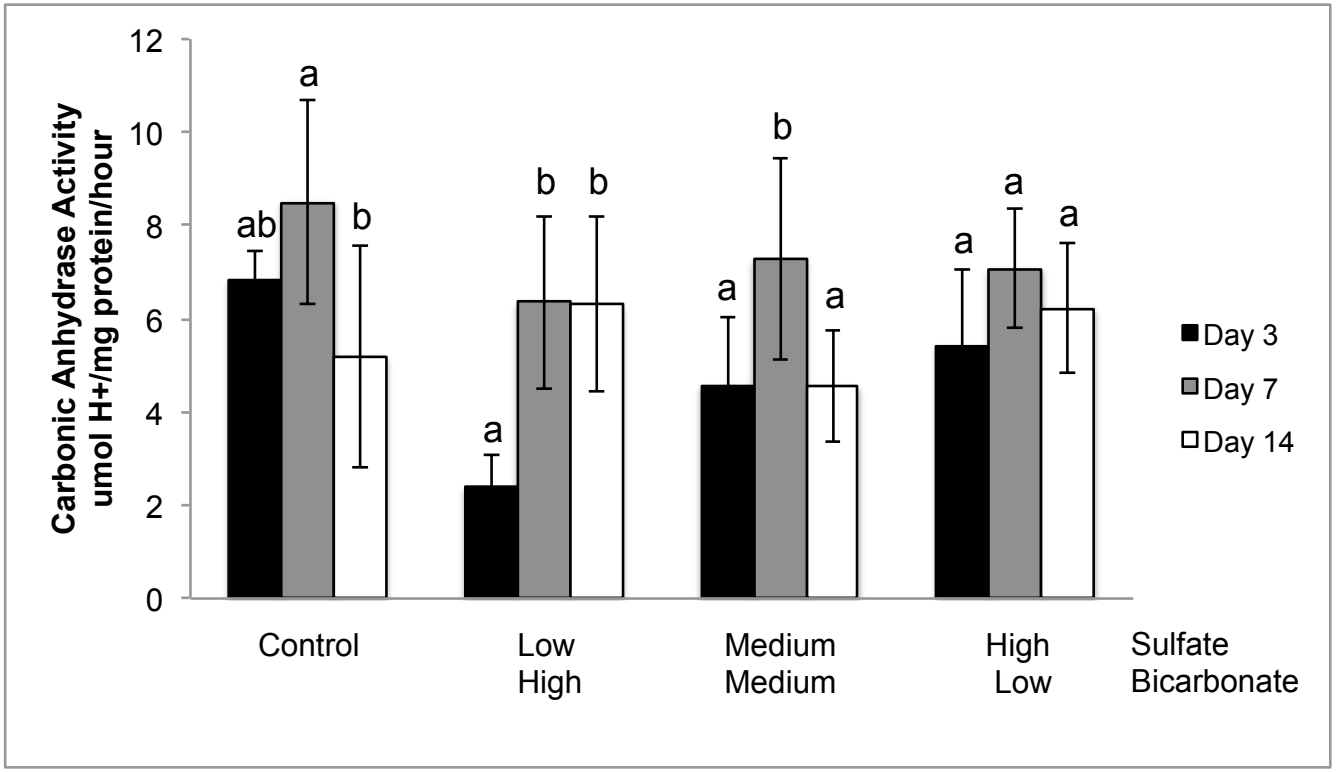

Figure 5.7. Carbonic anhydrase activity in *P. promelas* gill tissue following sulfate:bicarbonate mixture exposures.

Bars represent average carbonic anhydrase activity measured as μmol H^+/mg protein/hour ± 95% confidence intervals (α = 0.05). Significant differences within treatments are indicated by different letters derived from Fisher's LSD (*p* value ≤ 0.05). Error bars with the same letter indicate no significant difference.

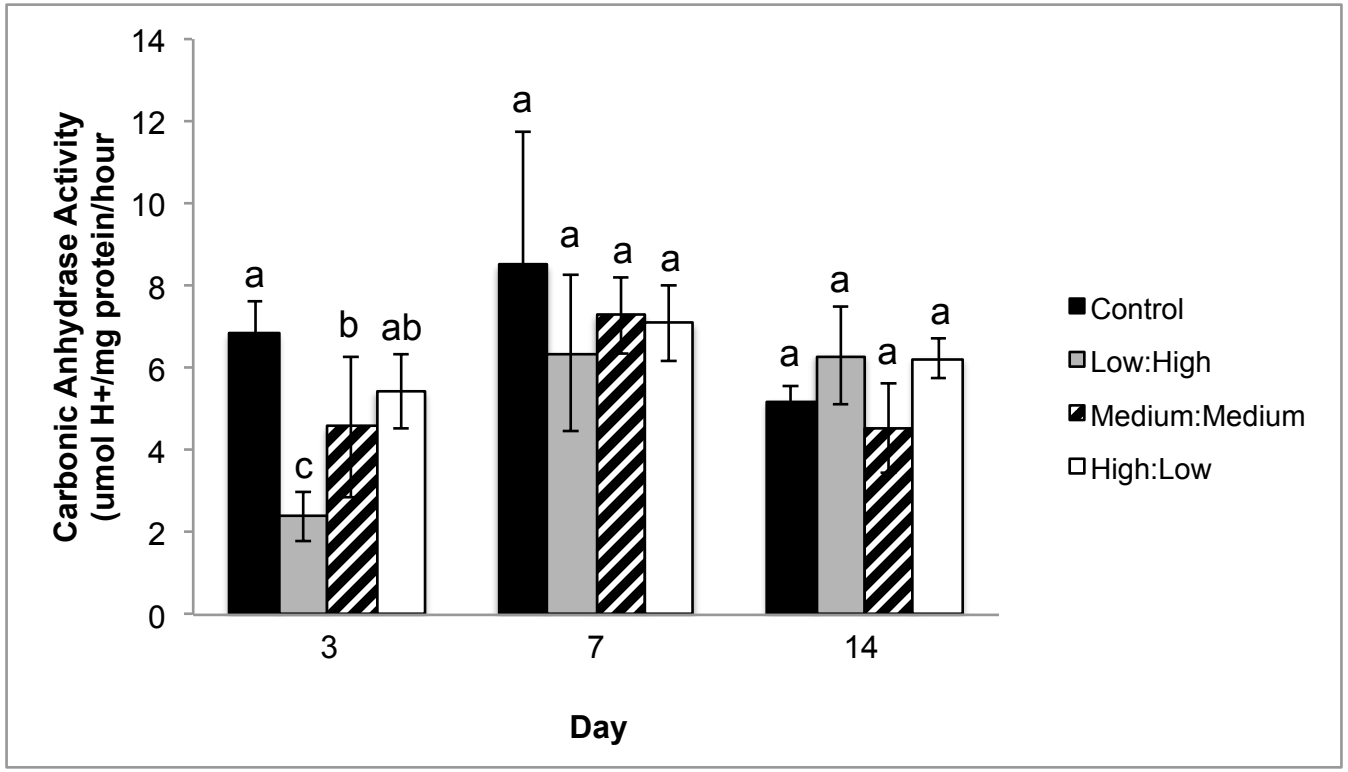

Figure 5.8. Changes in carbonic anhydrase activity in *P. promelas* gill tissue following sulfate:bicarbonate mixture exposures. Bars represent average carbonic anhydrase activity measured as μmol H^+/mg protein/hour ± 95% confidence intervals ($\alpha = 0.05$). Significant differences within sampling days are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

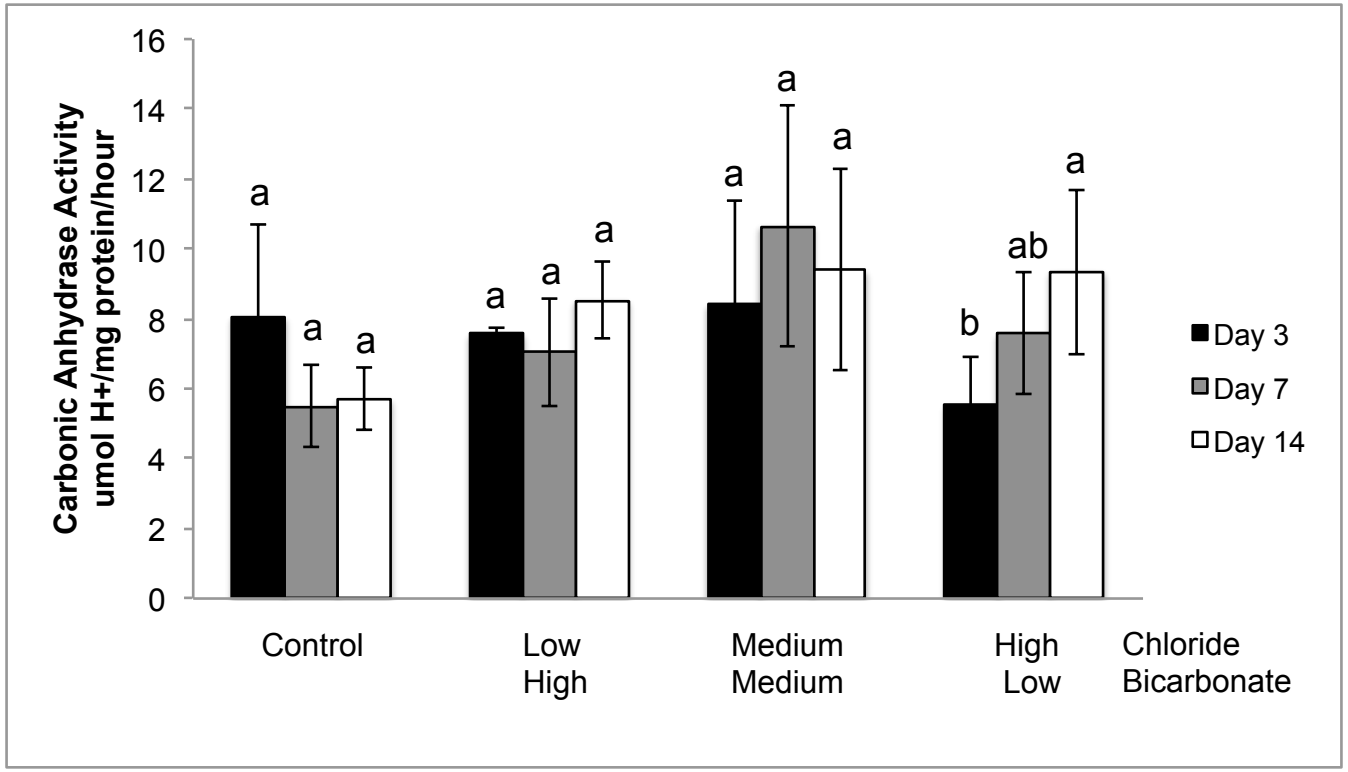

Figure 5.9. Carbonic anhydrase activity in *P. promelas* gill tissue following chloride:bicarbonate exposures. Bars represent average carbonic anhydrase activity measured as μmol H^+/mg protein/hour ± 95% confidence intervals ($\alpha = 0.05$). Significant differences within treatments are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

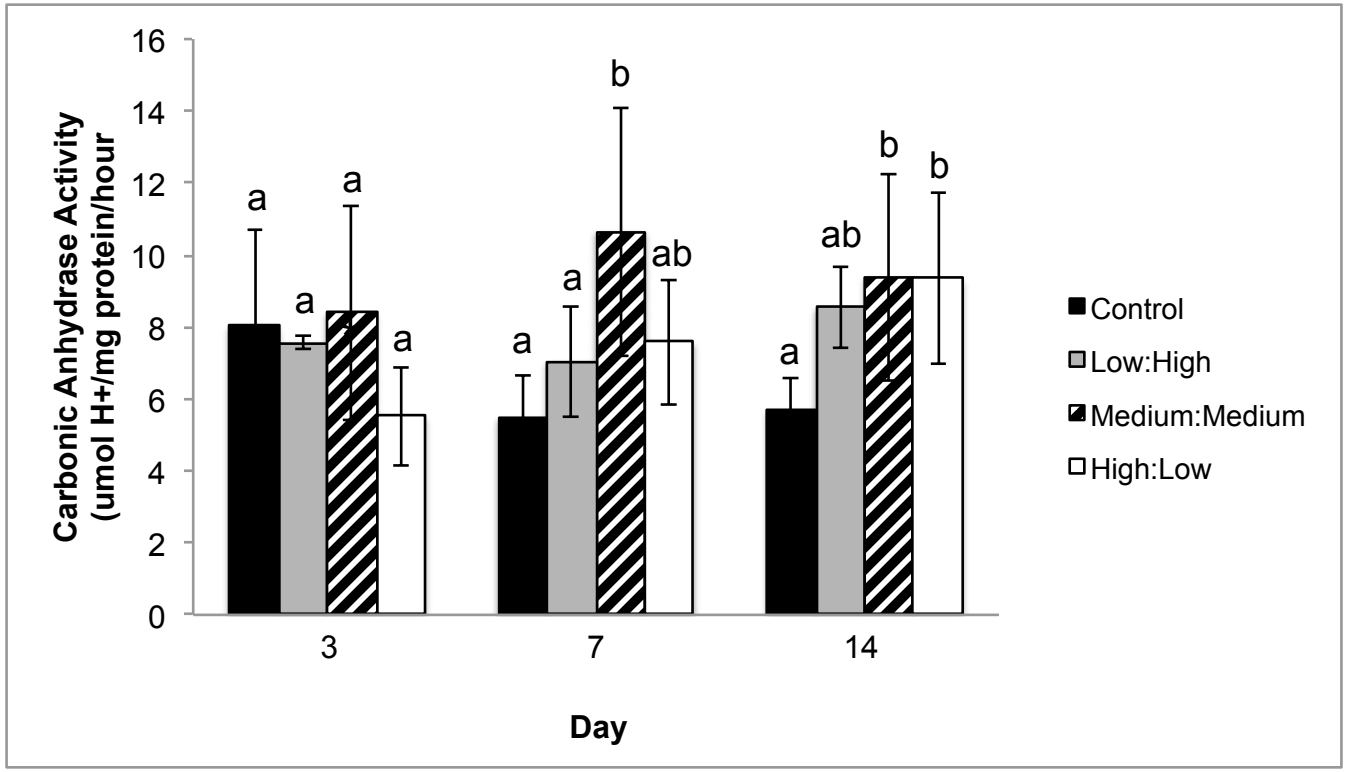

Figure 5.10. Changes in carbonic anhydrase activity in *P. promelas* gill tissue following chloride:bicarbonate mixture exposures. Bars represent average carbonic anhydrase activity measured as μmol H^+/mg protein/hour ± 95% confidence intervals ($\alpha = 0.05$). Significant differences within sampling days are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

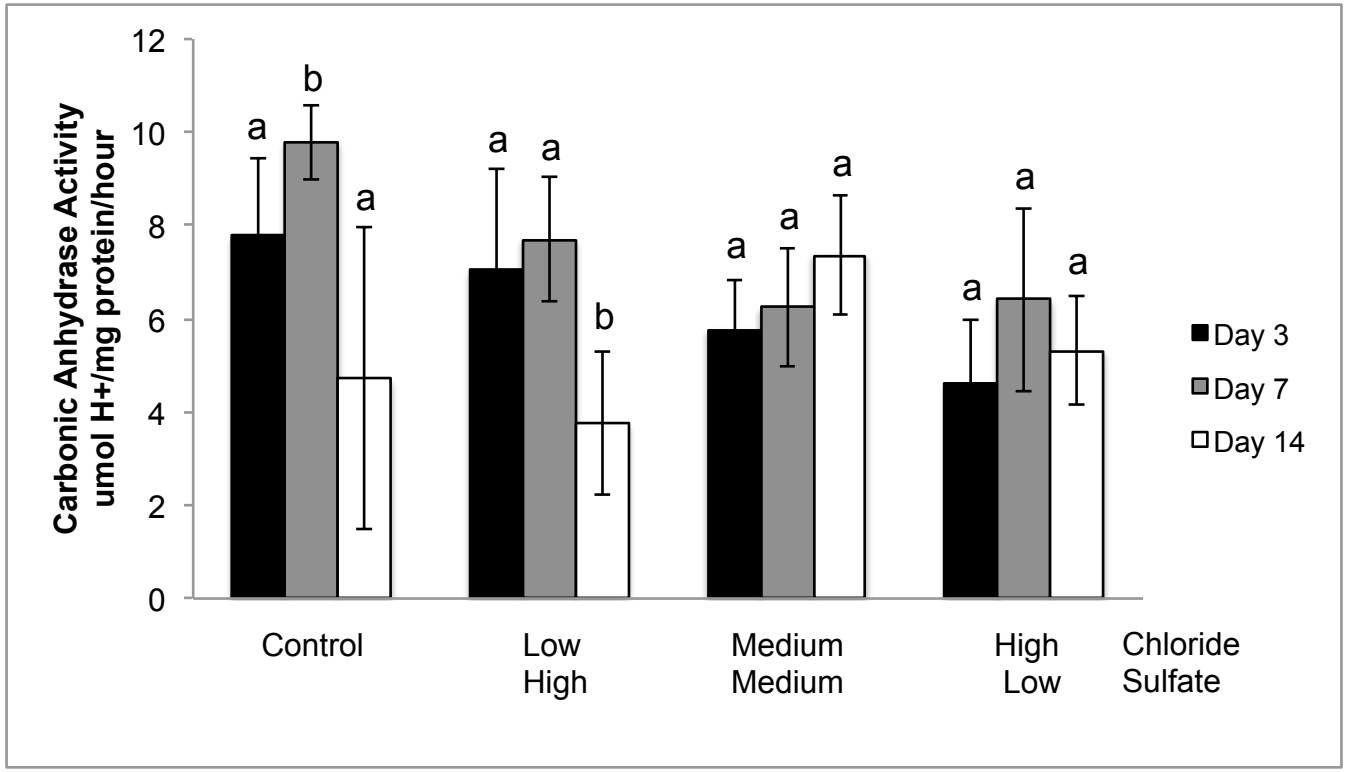

Figure 5.11. Carbonic anhydrase activity in *P. promelas* gill tissue following chloride:sulfate mixture exposures. Bars represent average carbonic anhydrase activity measured as μmol H^+/mg protein/hour ± 95% confidence intervals ($\alpha = 0.05$). Significant differences within treatments are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

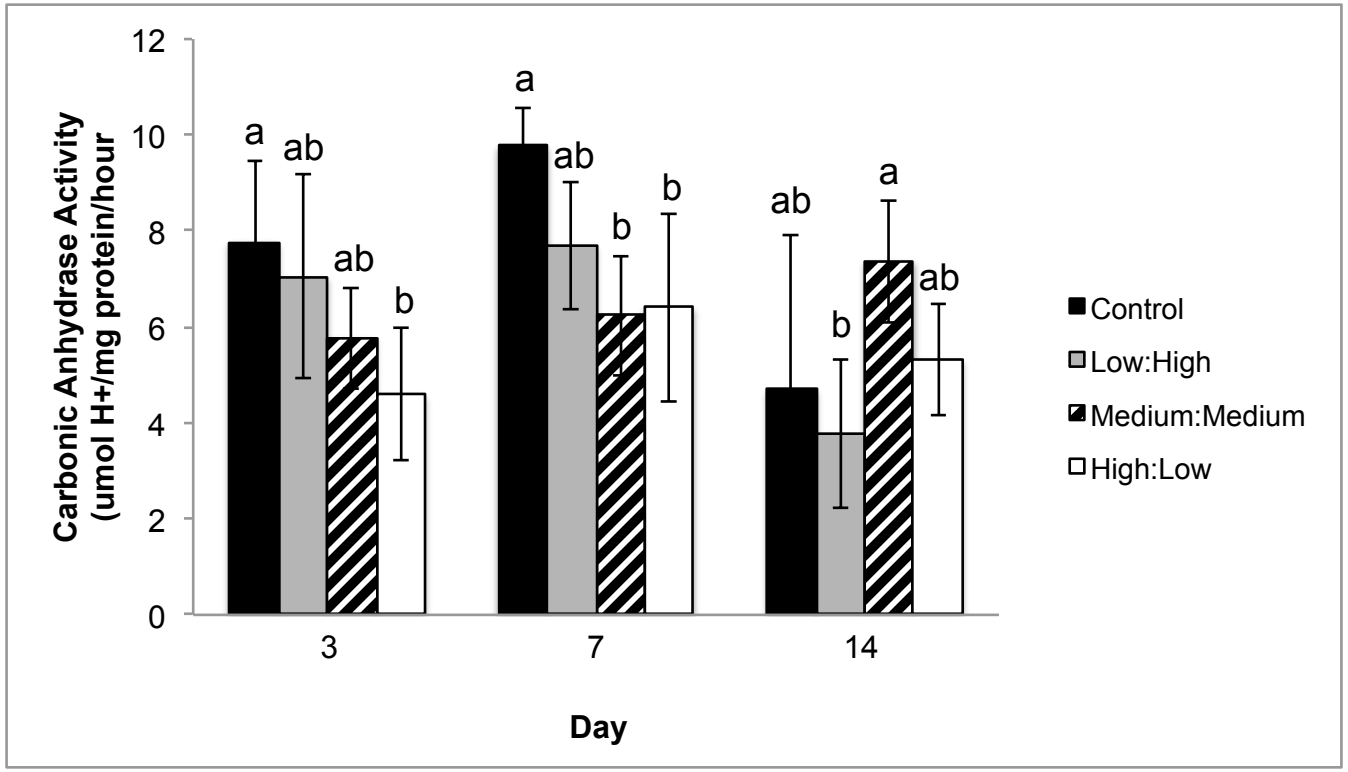

Figure 5.12. Changes in carbonic anhydrase activity in *P. promelas* gill tissue chloride:sulfate mixture exposures. Bars represent average carbonic anhydrase activity measured as μmol H^+/mg protein/hour ± 95% confidence intervals ($\alpha = 0.05$). Significant differences within sampling days are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

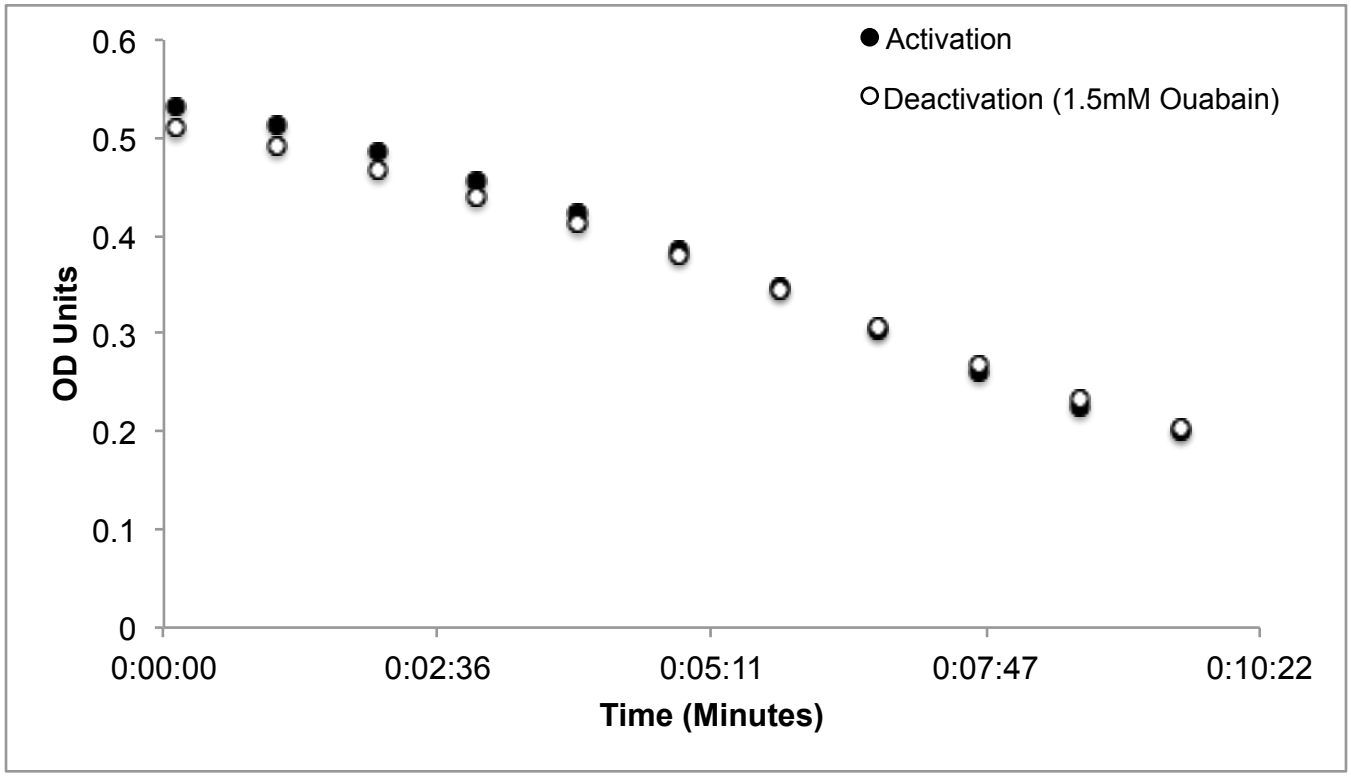

Figure 5.13. Preliminary Results: The lack of inhibition of Na^+/K^+-ATPase by 1.5 mM ouabain in *P. promelas* gill tissue.

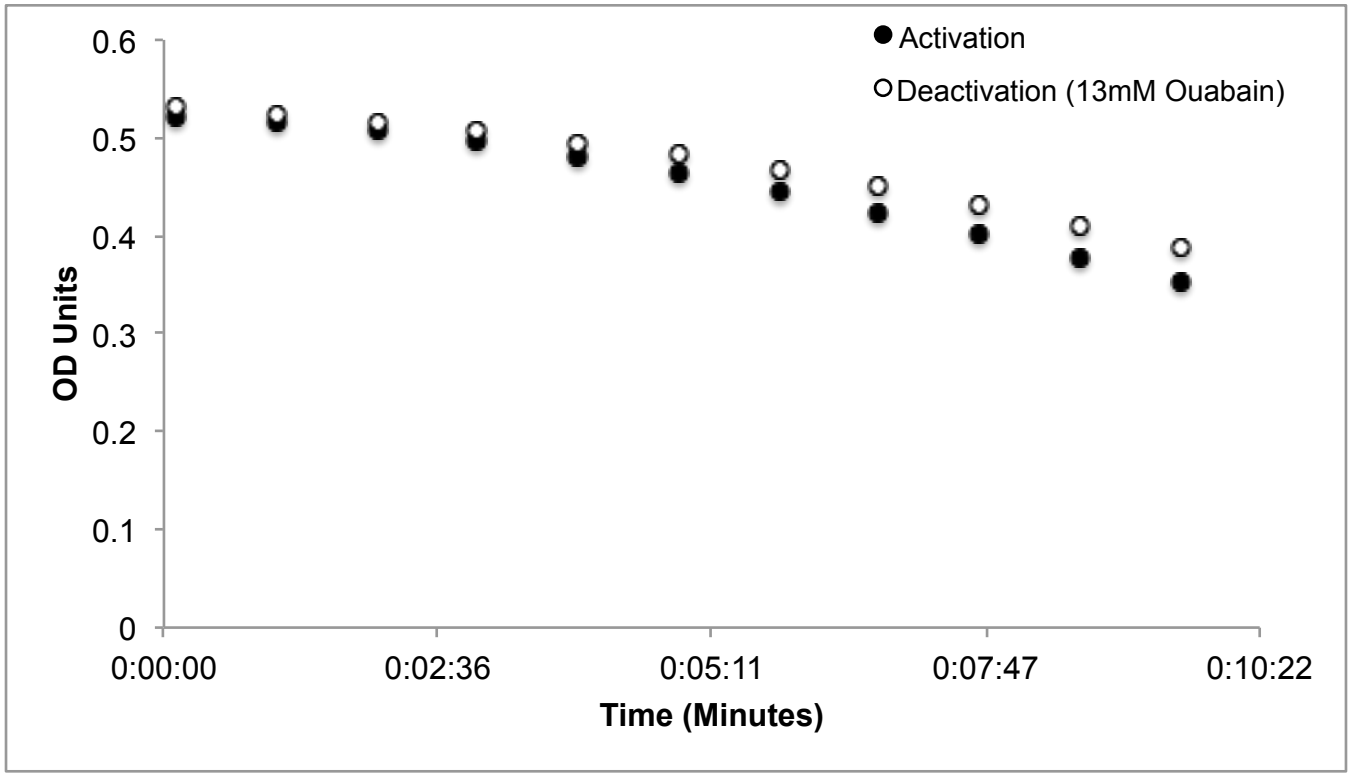

Figure 5.14. Preliminary Results: The lack of inhibition of Na^+/K^+-ATPase by 13.0 mM ouabain in *P. promelas* gill tissue.

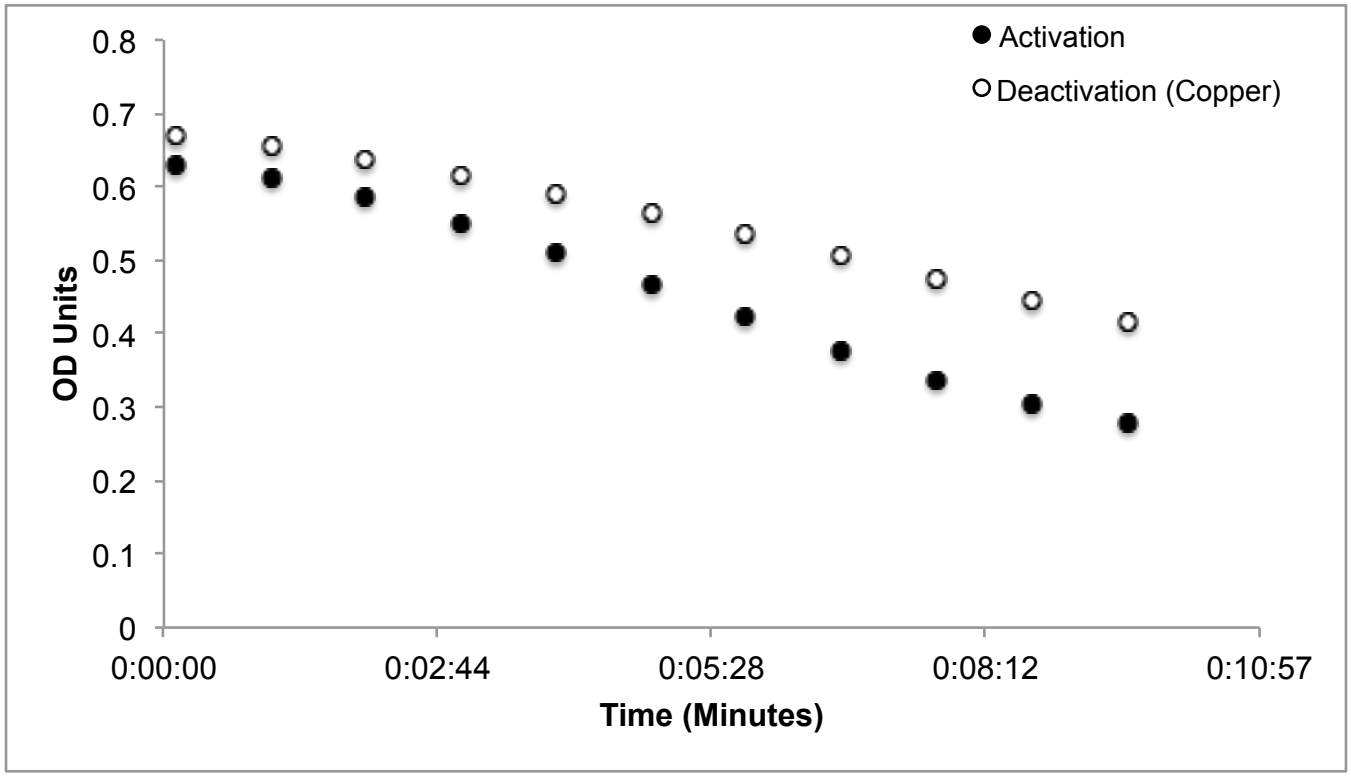

Figure 5.15. Preliminary Results: The inhibition of Na^+/K^+-ATPase by 157 mM copper in *P. promelas* gill tissue.

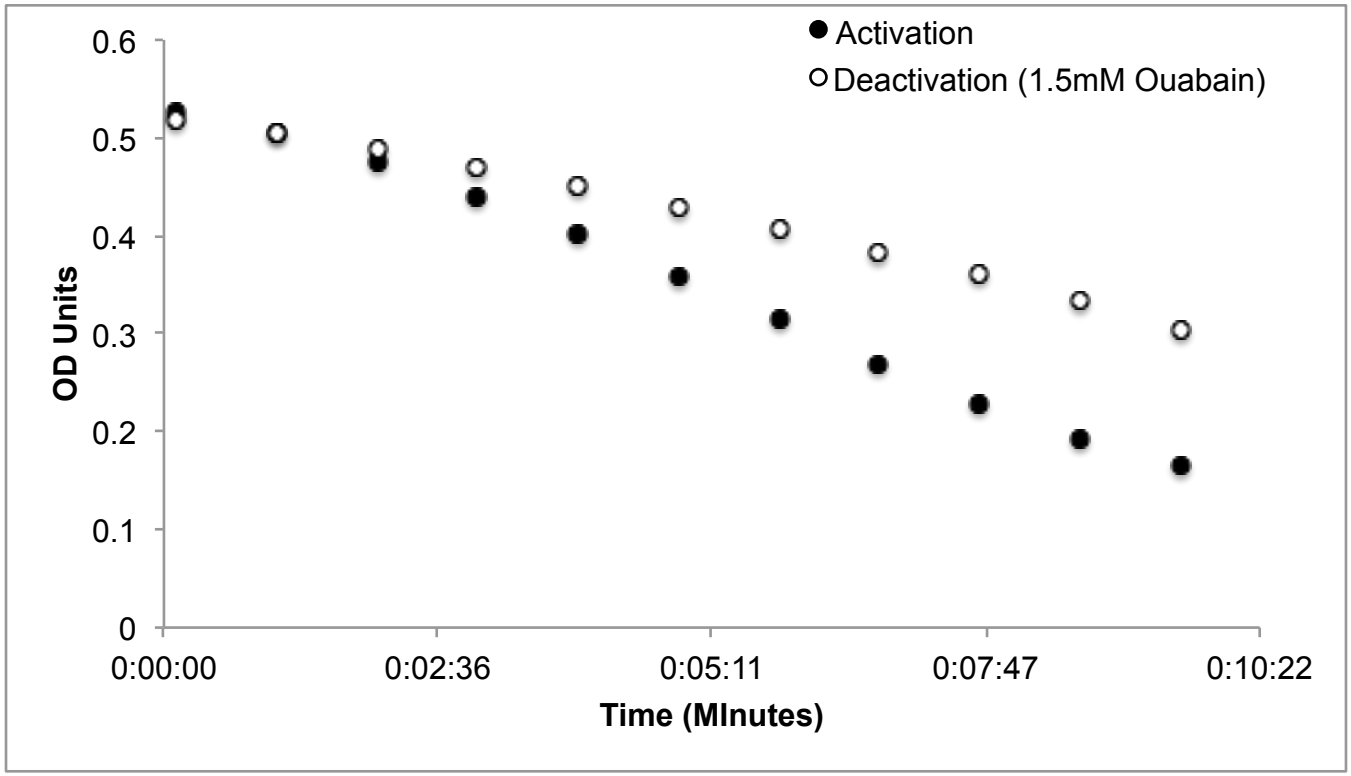

Figure 5.16. Preliminary Results: The inhibition of Na^+/K^+-ATPase by 1.5 mM ouabain in *F. heteroclitus* gill tissue.

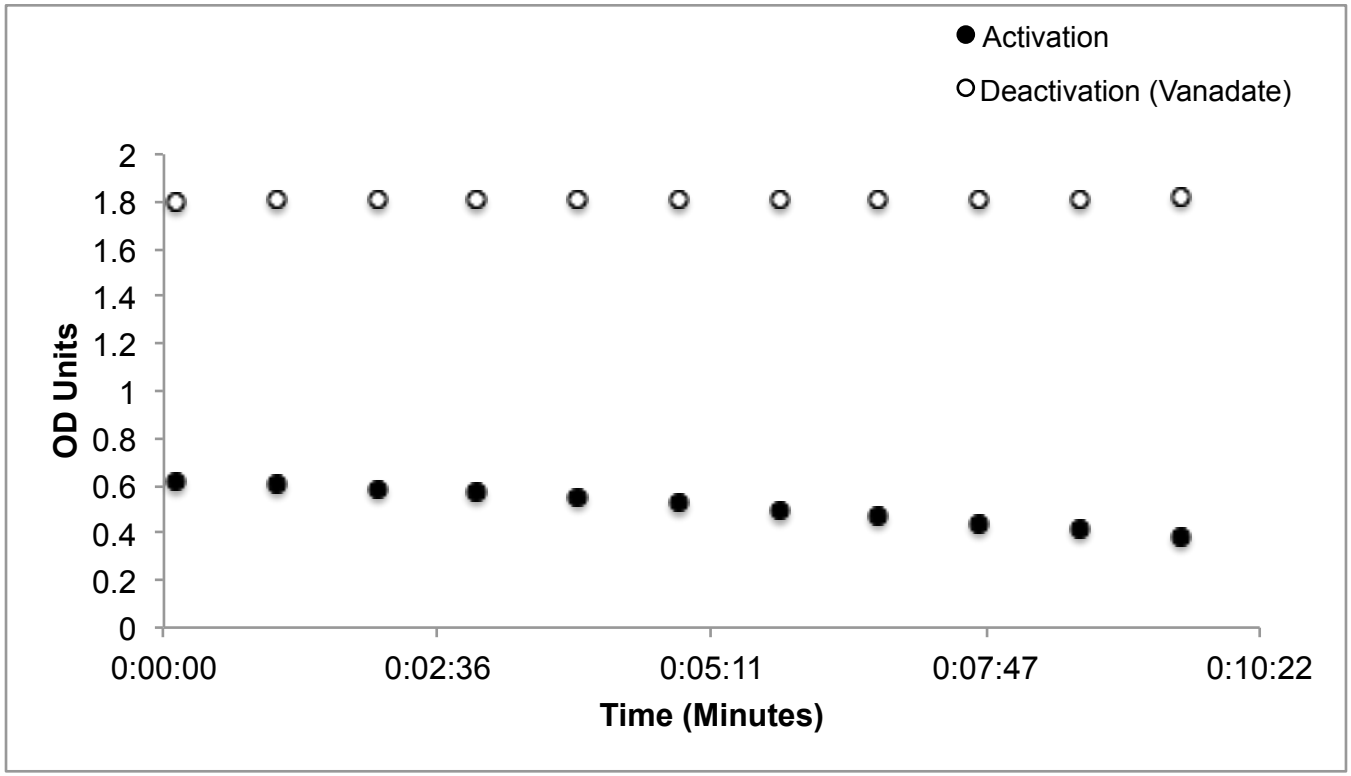

Figure 5.17. Preliminary Results: The inhibition of Na^+/K^+-ATPase by 10.0 mM *ortho*vanadate in *P. promelas* gill tissue.

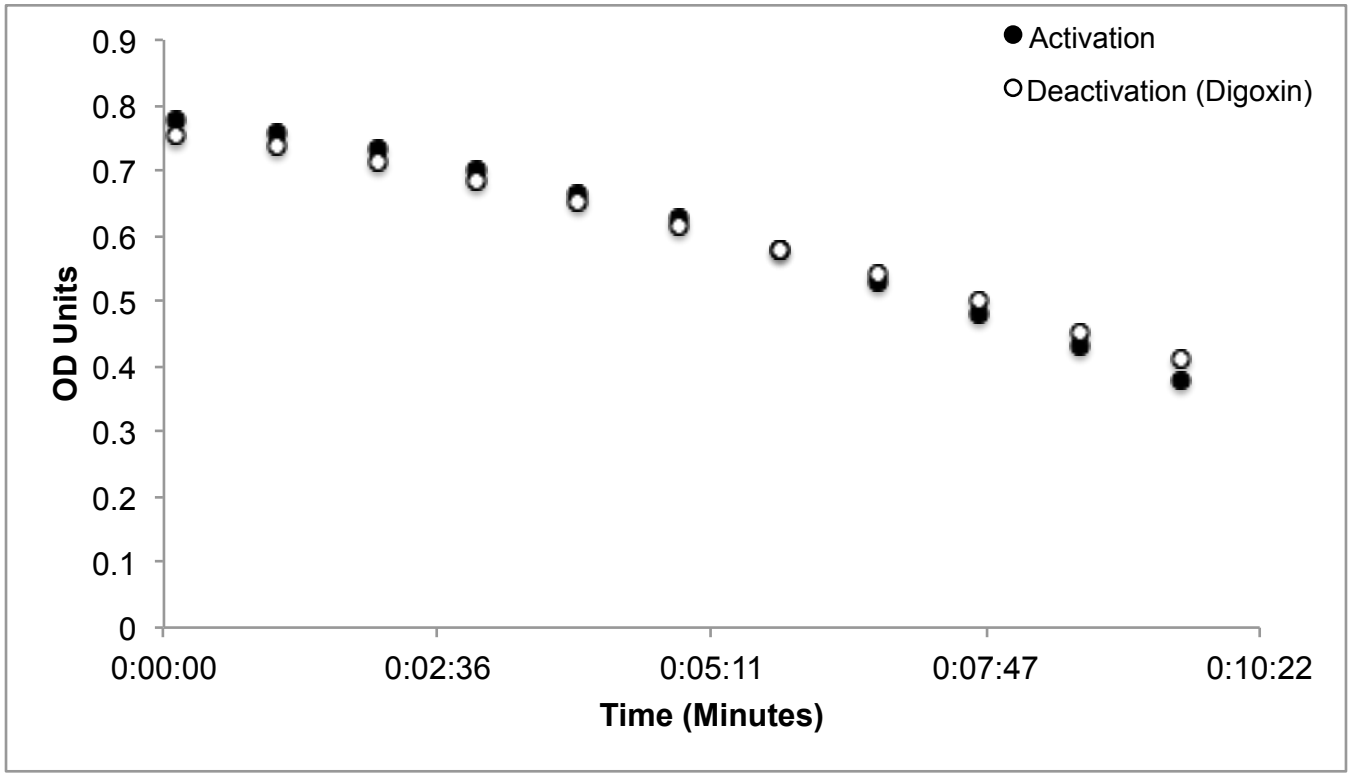

Figure 5.18. Preliminary Results: The lack of inhibition of Na^+/K^+-ATPase by 6.5 mM digoxin in *P. promelas* gill tissue.

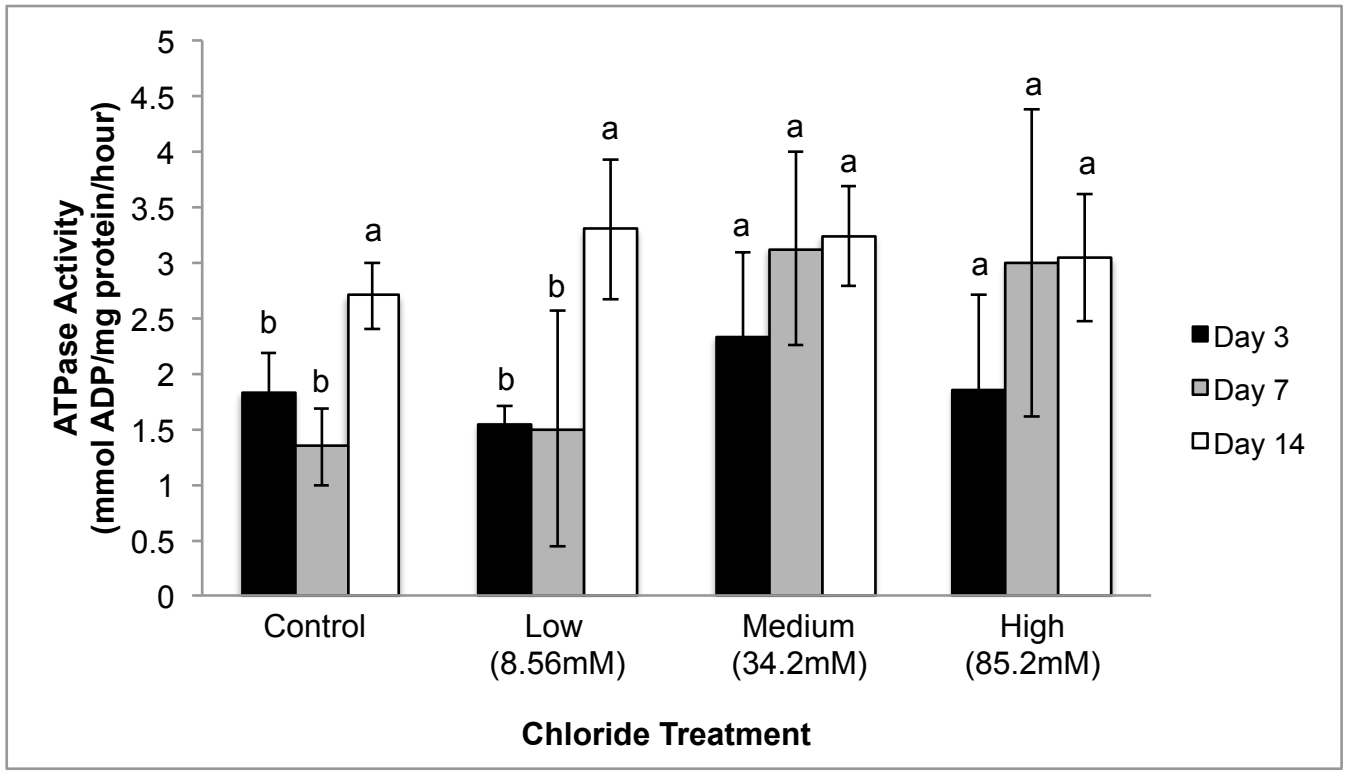

Figure 5.19. Total ATPase activity in *P. promelas* gill tissue chloride-only exposures. Bars represent average ATPase activity measured as mmol ADP/mg protein/hour ± 95% confidence intervals ($\alpha = 0.05$). Significant differences within treatments are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

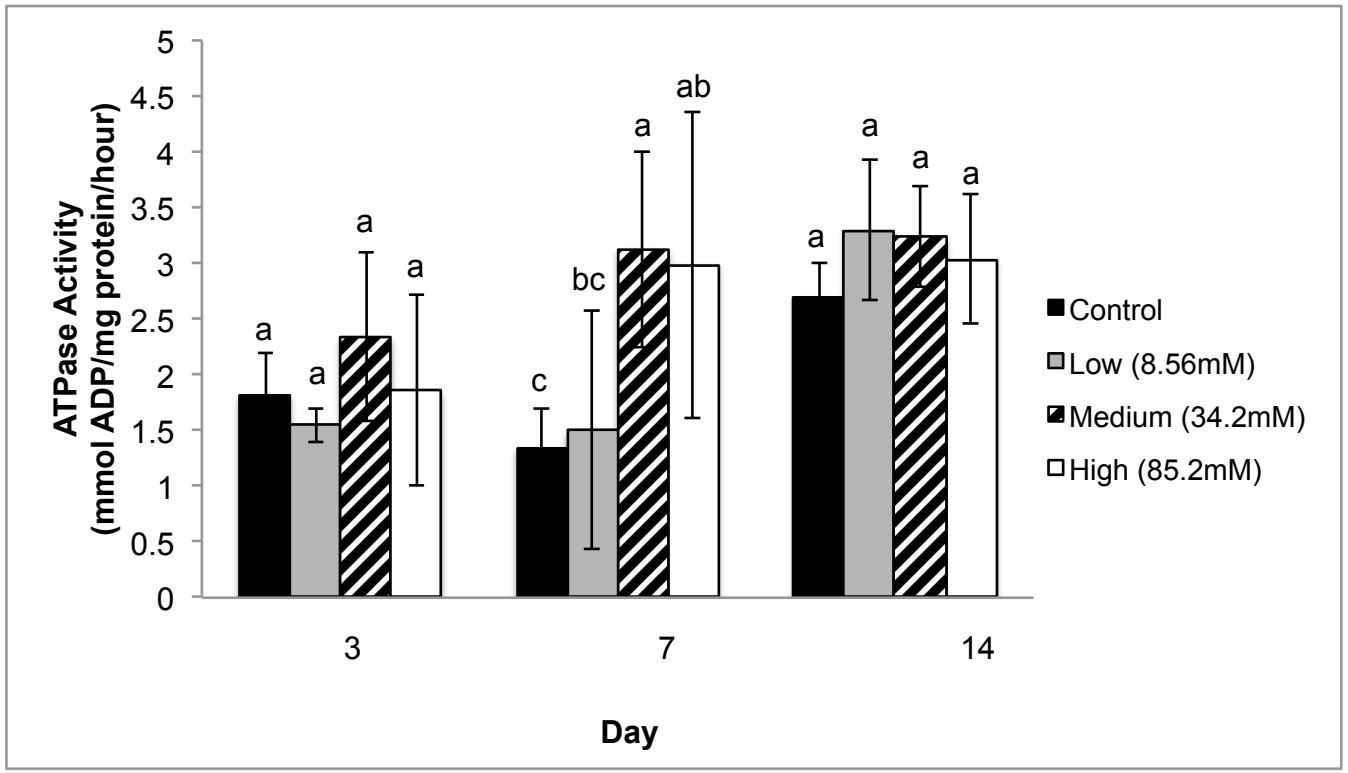

Figure 5.20. Changes in total ATPase activity in *P. promelas* gill tissue chloride-only exposures. Bars represent average ATPase activity measured as mmol ADP/mg protein/hour ± 95% confidence intervals ($\alpha = 0.05$). Significant differences within sampling days are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

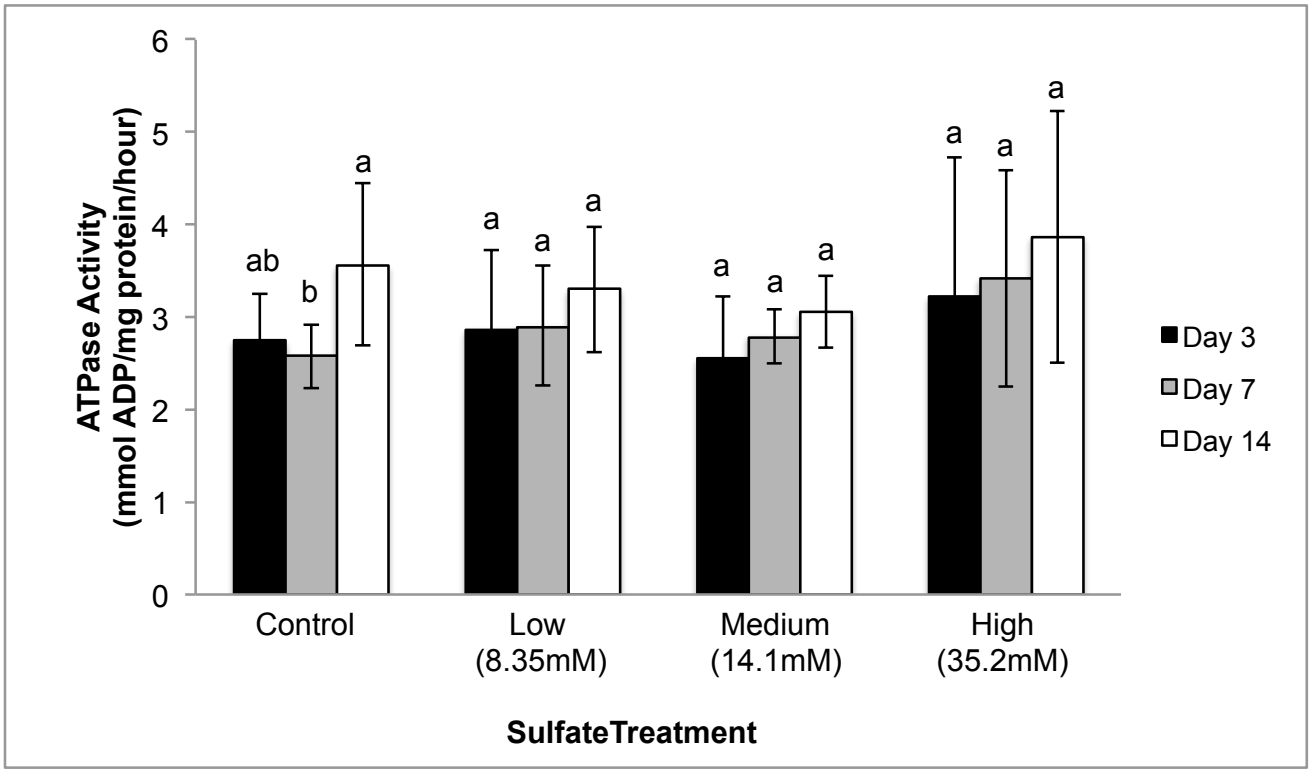

Figure 5.21. Total ATPase activity in *P. promelas* gill tissue sulfate-only exposures. Bars represent average ATPase activity measured as mmol ADP/mg protein/hour ± 95% confidence intervals ($\alpha = 0.05$). Significant differences within treatments are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

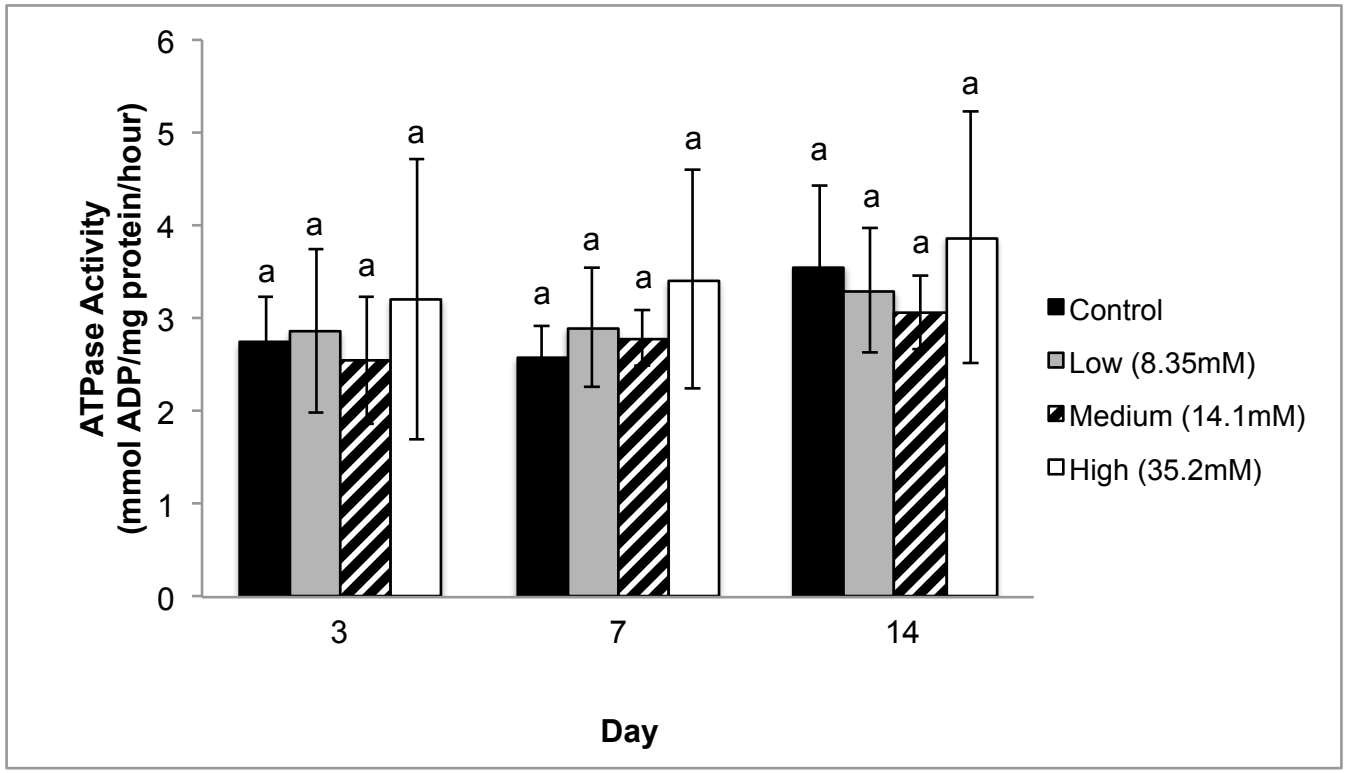

Figure 5.22. Changes in total ATPase activity in *P. promelas* gill tissue following sulfate-only exposures. Bars represent average ATPase activity measured as mmol/mg protein/hour ± 95% confidence intervals ($\alpha = 0.05$). Significant differences within sampling days are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

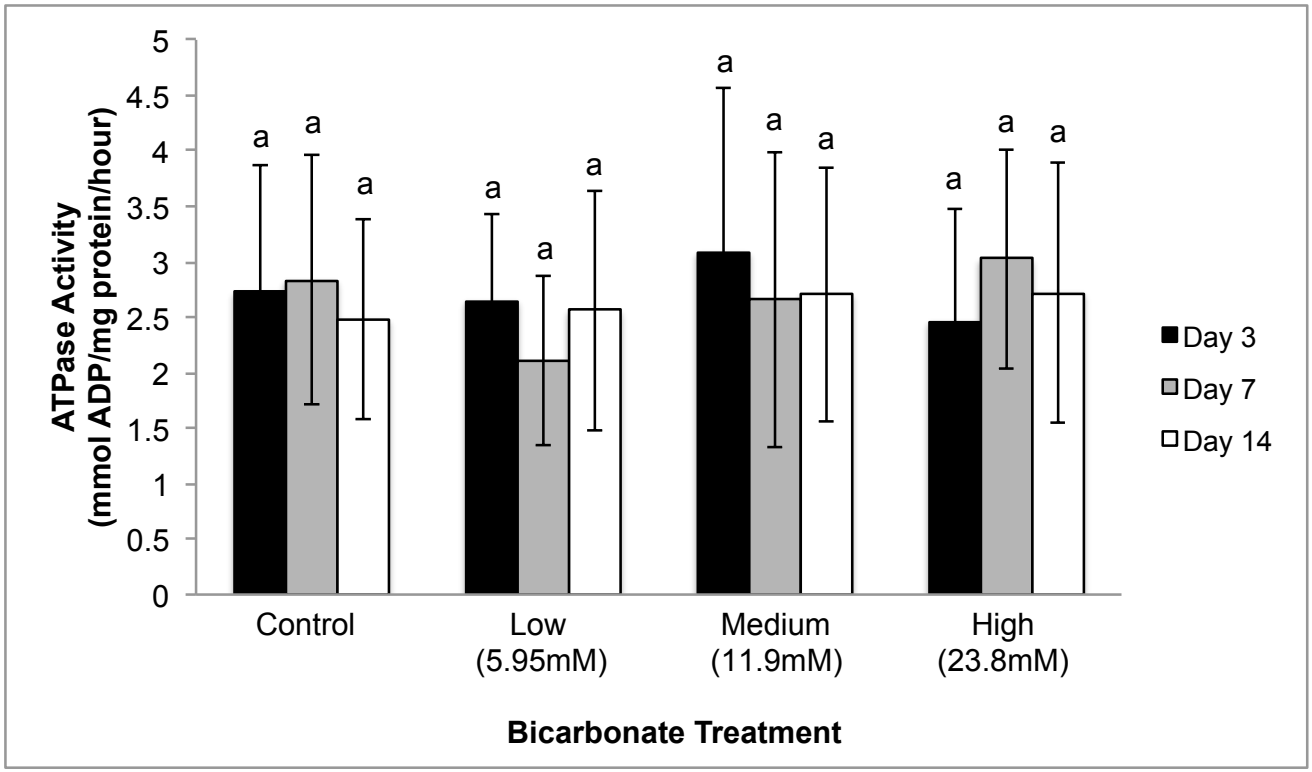

Figure 5.23. Total ATPase activity in *P. promelas* gill tissue following bicarbonate-only exposures. Bars represent average ATPase activity measured as mmol ADP/mg protein/hour ± 95% confidence intervals ($\alpha = 0.05$). Significant differences within treatments are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

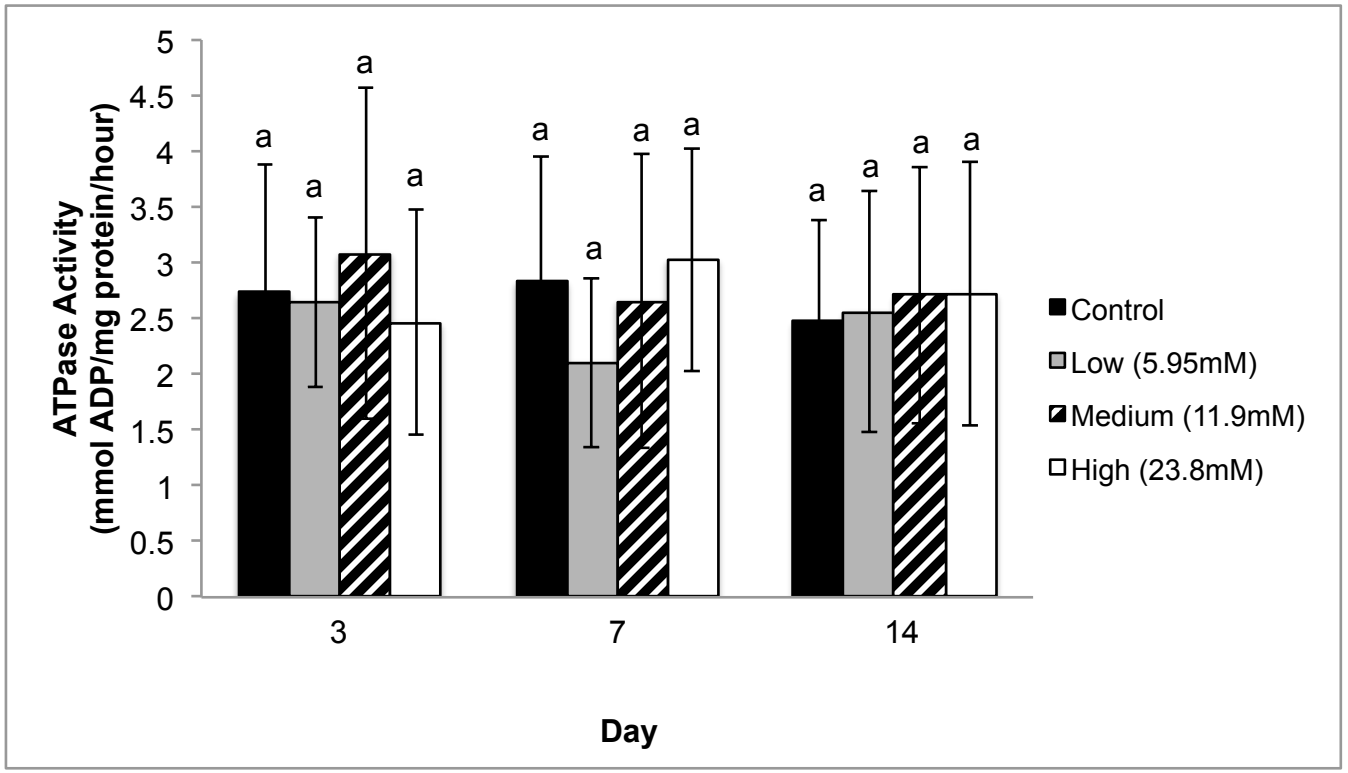

Figure 5.24. Changes in total ATPase activity in *P. promelas* gill tissue following bicarbonate-only exposures. Bars represent average ATPase activity measured as mmol ADP/mg protein/hour ± 95% confidence intervals ($\alpha = 0.05$). Significant differences within sampling days are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

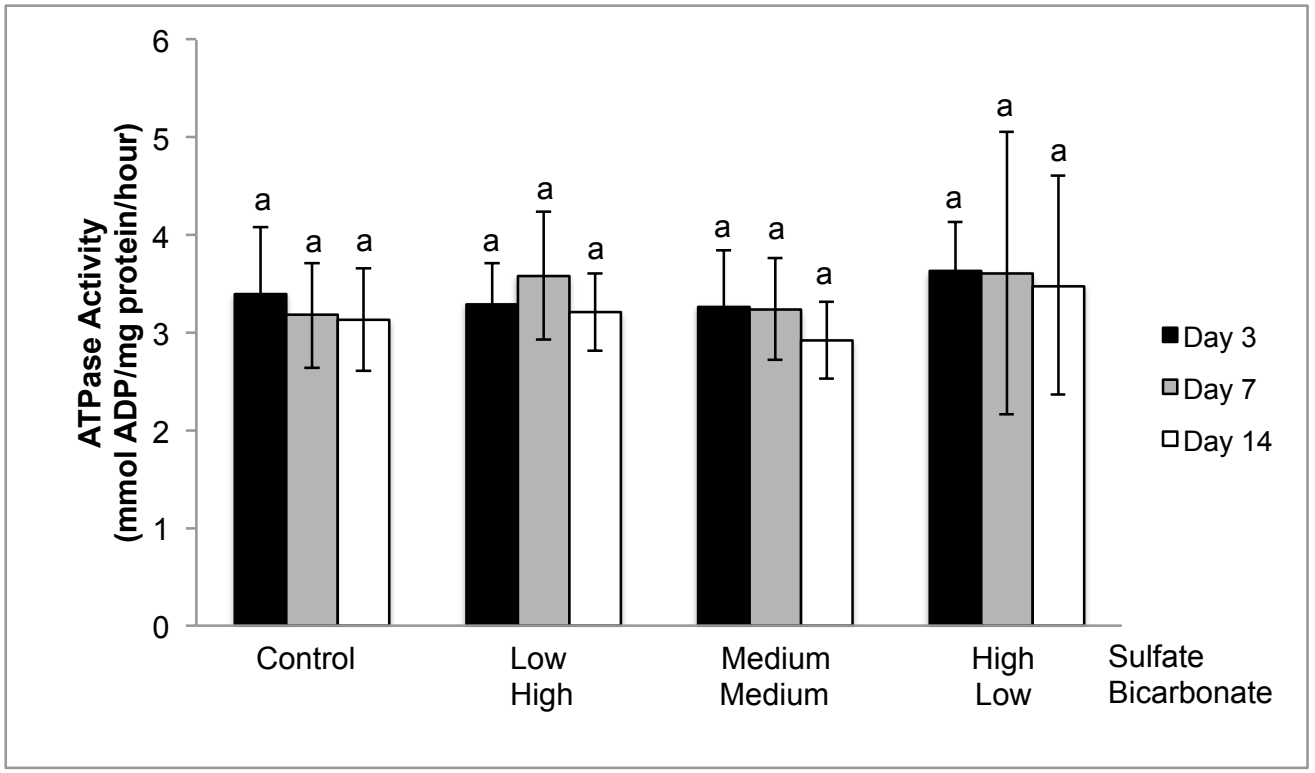

Figure 5.25. Total ATPase activity in *P. promelas* gill tissue following sulfate:bicarbonate mixture exposures. Bars represent average ATPase activity measured as mmol ADP/mg protein/hour ± 95% confidence intervals ($\alpha = 0.05$). Significant differences within treatments are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

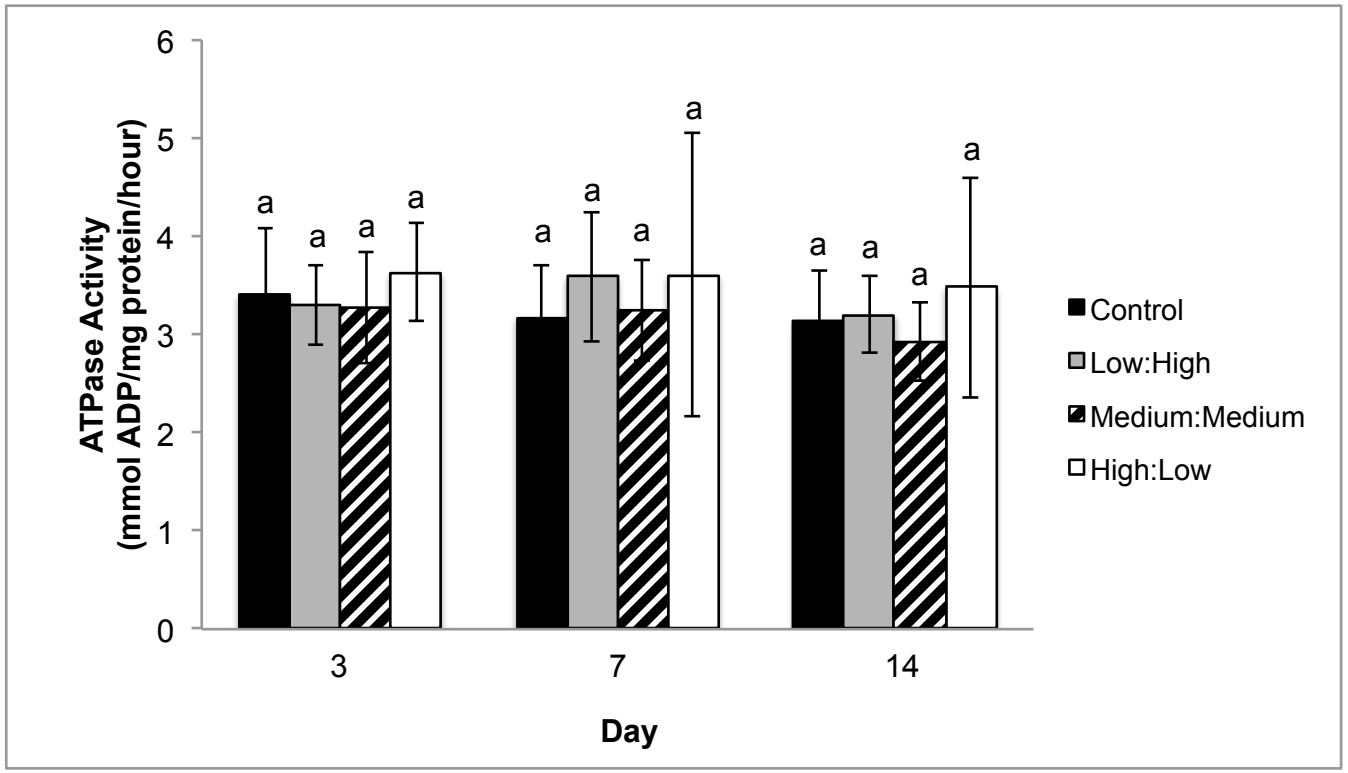

Figure 5.26. Changes in total ATPase activity in *P. promelas* gill tissue following sulfate:bicarbonate mixture exposures. Bars represent average ATPase activity measured as mmol ADP/mg protein/hour ± 95% confidence intervals (α = 0.05). Significant differences within sampling days are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

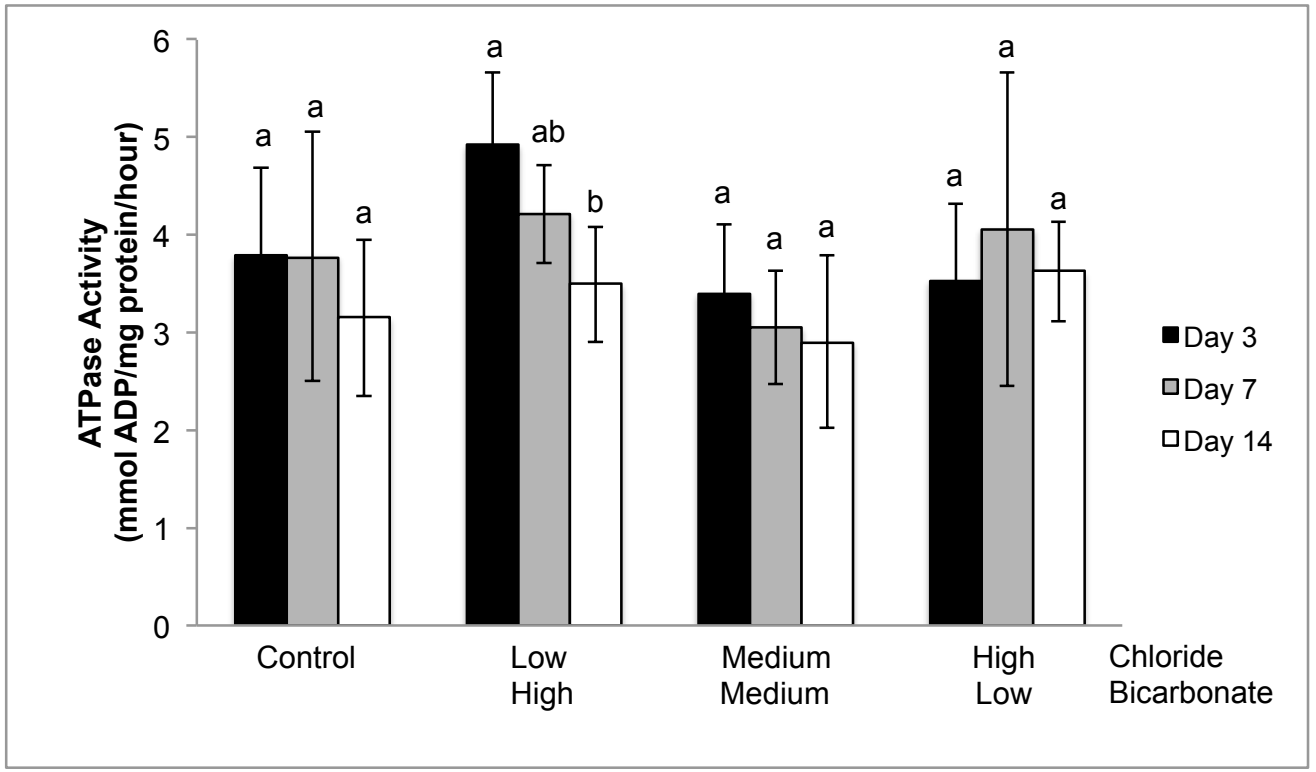

Figure 5.27. Total ATPase activity in *P. promelas* gill tissue following chloride:bicarbonate mixture exposures. Bars represent average ATPase activity measured as mmol ADP/mg protein/hour ± 95% confidence intervals ($\alpha = 0.05$). Significant differences within treatments are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

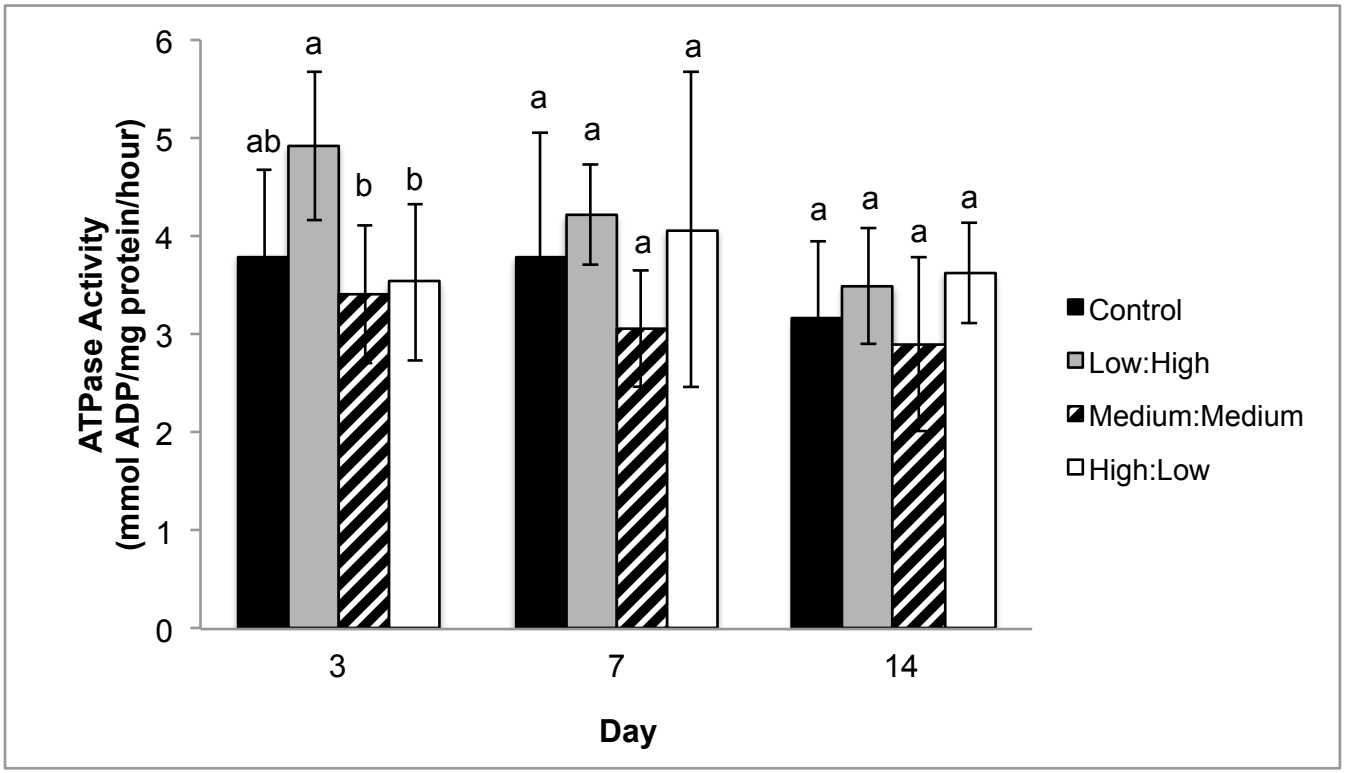

Figure 5.28. Changes in total ATPase activity in *P. promelas* gill tissue following chloride:bicarbonate mixture exposures. Bars represent average ATPase activity measured as mmol ADP/mg protein/hour ± 95% confidence intervals (α = 0.05). Significant differences within sampling days are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

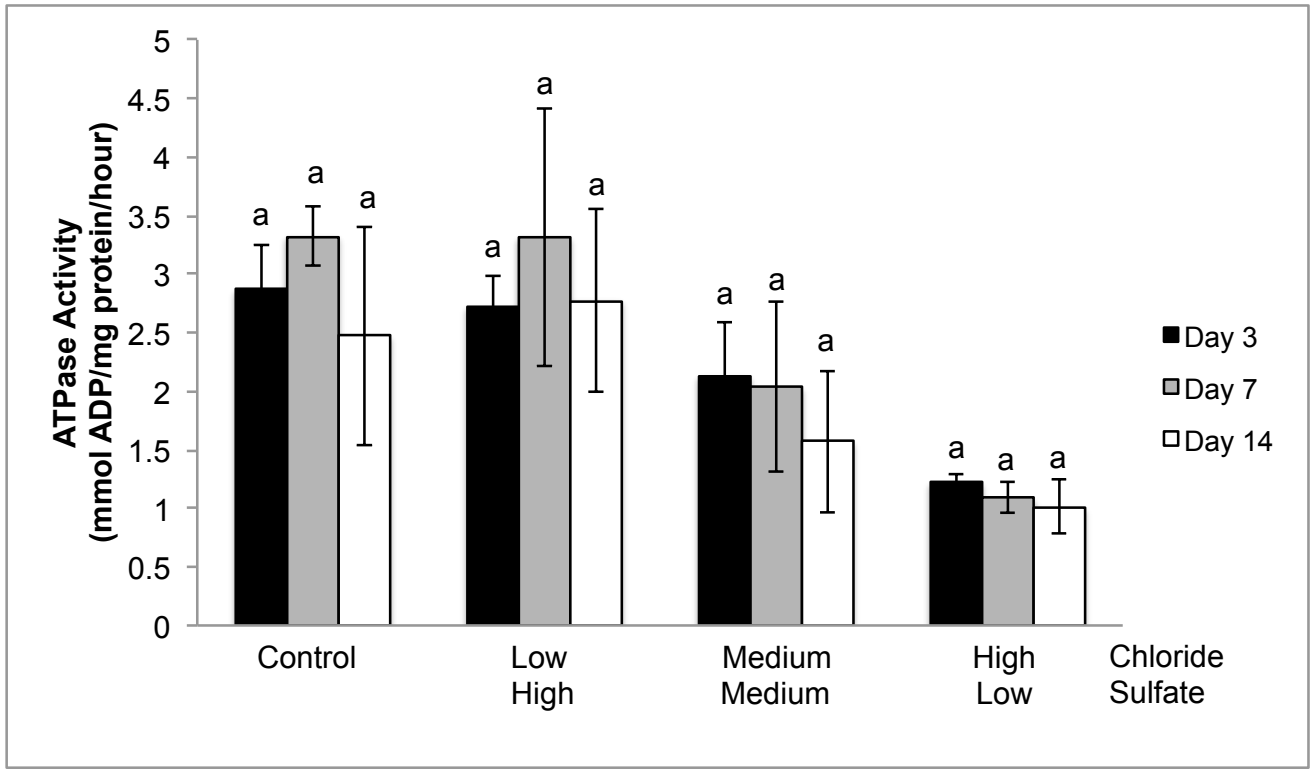

Figure 5.29. Total ATPase activity in *P. promelas* gill tissue following chloride:sulfate mixture exposures. Bars represent average ATPase activity measured as mmol ADP/mg protein/hour ± 95% confidence intervals ($\alpha = 0.05$). Significant differences within treatments are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

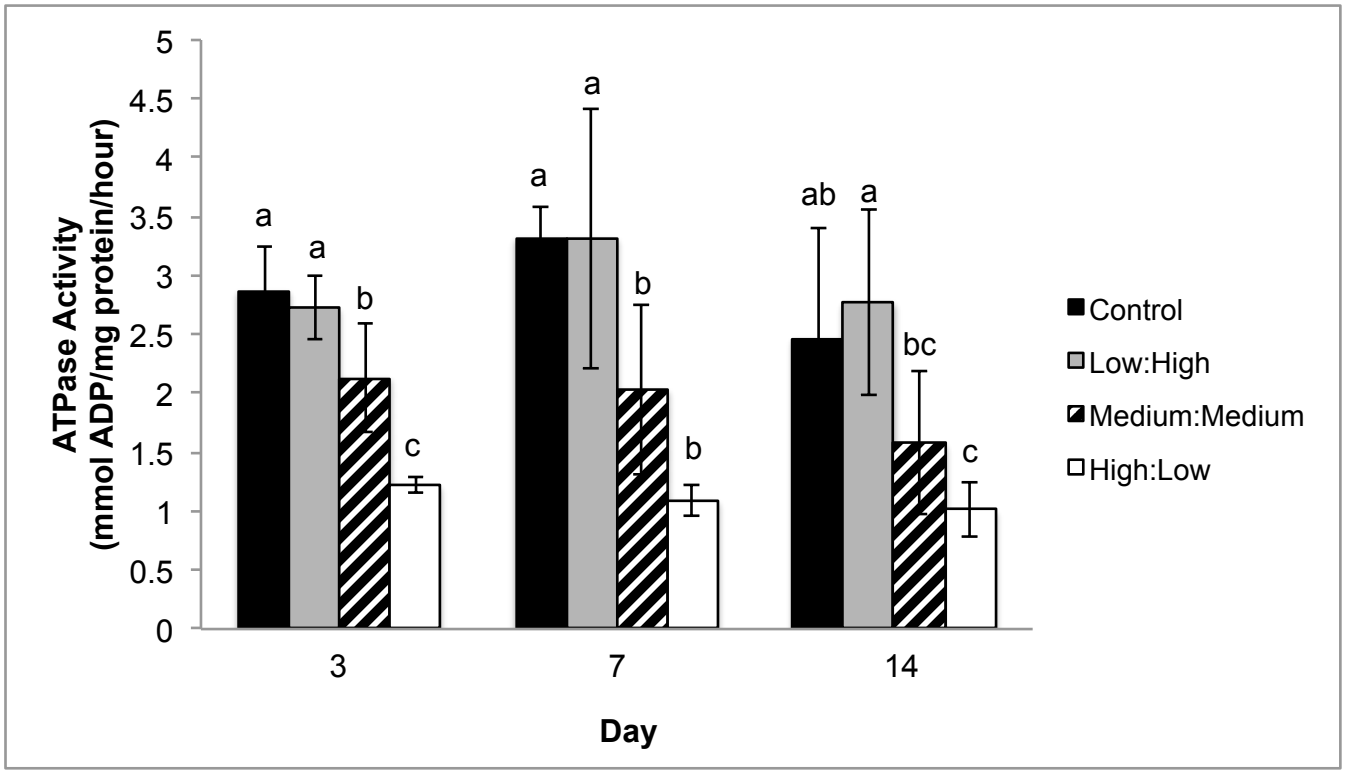

Figure 5.30. Changes in total ATPase activity in *P. promelas* gill tissue following chloride:sulfate mixture exposures.

Bars represent average ATPase activity measured as mmol ADP/mg protein/hour ± 95% confidence intervals ($\alpha = 0.05$). Significant differences within sampling days are indicated by different letters derived from Fisher's LSD (p value ≤ 0.05). Error bars with the same letter indicate no significant difference.

Table 5.1. *p* values for changes in carbonic anhydrase activity following chloride-only exposures[a].

Chloride-Only	Control	Low	Medium	High
Control		Day 3: 0.4912 Day 7: 0.0685 Day 14: 0.1165	Day 3: 0.0885 Day 7: 0.0620 Day 14: 0.4605	Day 3: 0.2166 Day 7: 0.8424 Day 14: 0.4688
Low			Day 3: 0.2836 Day 7: 0.9580 Day 14: 0.0280*	Day 3: 0.5688 Day 7: 0.0990 Day 14: 0.3726
Medium				Day 3: 0.6049 Day 7: 0.0900 Day 14: 0.1535
High				

[a]Asterisk (*) indicates significant difference (*p* value ≤ 0.05).

Table 5.2 *p* values for changes in carbonic anhydrase activity following sulfate-only exposures[a].

Sulfate-Only	Control	Low	Medium	High
Control		Day 3: 0.3005 Day 7: 0.8411 Day 14: 0.1823	Day 3: 0.0599 Day 7: 0.992 Day 14: 0.9519	Day 3: 0.0868 Day 7: 0.0785 Day 14: 0.9564
Low			Day 3: 0.3539 Day 7: 0.8488 Day 14: 0.1649	Day 3: 0.4615 Day 7: 0.0536 Day 14: 0.1664
Medium				Day 3: 0.8439 Day 7: 0.0771 Day 14: 0.9955
High				

[a]Asterisk (*) indicates significant difference (*p* value ≤ 0.05).

Table 5.3. *p* values for changes in carbonic anhydrase activity following bicarbonate-only exposures[a].

Bicarbonate-Only	Control	Low	Medium	High
Control		Day 3: 0.0006* Day 7: 0.4821 Day 14: 0.0114*	Day 3: <0.0001* Day 7: 0.7132 Day 14: 0.0068*	Day 3: 0.0004* Day 7: 0.0469* Day 14: 0.0049*
Low			Day 3: 0.3398 Day 7: 0.7341 Day 14: 0.8064	Day 3: 0.8750 Day 7: 0.0111* Day 14: 0.6937
Medium				Day 3: 0.4219 Day 7: 0.0224* Day 14: 0.8812
High				

[a]Asterisk (*) indicates significant difference (*p* value ≤ 0.05).

Table 5.4. *p* values for changes in carbonic anhydrase activity following sulfate:bicarbonate mixture exposures[a].

Sulfate:Bicarbonate	Control	Low:High	Medium:Medium	High:Low
Control		Day 3: <0.0001* Day 7: 0.1376 Day 14: 0.3993	Day 3: 0.0172* Day 7: 0.3874 Day 14: 0.6218	Day 3: 0.1187 Day 7: 0.3183 Day 14: 0.4305
Low:High			Day 3: 0.0219* Day 7: 0.5095 Day 14: 0.1899	Day 3: 0.0027* Day 7: 0.6012 Day 14: 0.9552
Medium:Medium				Day 3: 0.3281 Day 7: 0.8893 Day 14: 0.2081
High:Low				

[a]Asterisk (*) indicates significant difference (*p* value ≤ 0.05).

Table 5.5. *p* values for changes in carbonic anhydrase activity following chloride:bicarbonate mixture exposures[a].

Chloride:Bicarbonate	Control	Low:High	Medium:Medium	High:Low
Control		Day 3: 0.7554 Day 7: 0.3374 Day 14: 0.0669	Day 3: 0.8183 Day 7: 0.0044* Day 14: 0.02098	Day 3: 0.1179 Day 7: 0.1984 Day 14: 0.0223*
Low:High			Day 3: 0.5896 Day 7: 0.0338* Day 14: 0.5598	Day 3: 0.2004 Day 7: 0.7288 Day 14: 0.5802
Medium:Medium				Day 3: 0.0776 Day 7: 0.0666 Day 14: 0.9757
High:Low				

[a]Asterisk (*) indicates significant difference (p value ≤ 0.05).

Table 5.6. *p* values for changes in carbonic anhydrase activity following chloride:sulfate mixture exposures[a].

Chloride:Sulfate	Control	Low:High	Medium:Medium	High:Low
Control		Day 3: 0.5409 Day 7: 0.0557 Day 14: 0.5231	Day 3: 0.1034 Day 7: 0.0029* Day 14: 0.0830	Day 3: 0.0144* Day 7: 0.0041* Day 14: 0.6748
Low:High			Day 3: 0.2867 Day 7: 0.1658 Day 14: 0.0236*	Day 3: 0.0501 Day 7: 0.2192 Day 14: 0.2960
Medium:Medium				Day 3: 0.3243 Day 7: 0.8648 Day 14: 0.1744
High:Low				

[a]Asterisk (*) indicates significant difference (*p* value ≤ 0.05).

Table 5.7. *p* values for carbonic anhydrase response between treatments following chloride-only exposures[a].

Chloride	Day 3	Day 7	Day 14
Day 3		Control: 0.3966 Low: 0.3926 Medium: 0.3295 High: 0.9381	Control: 0.4841 Low: 0.0374* Medium: 0.2944 High: 0.0149*
Day 7			Control: 0.1353 Low: 0.0073* Medium: 0.0562 High: 0.0173*
Day 14			

[a]Asterisk (*) indicates significant difference (*p* value ≤ 0.05).

Table 5.8. *p* values for carbonic anhydrase response between treatments following sulfate-only exposures[a].

Sulfate	Day 3	Day 7	Day 14
Day 3		Control: 0.4927 Low: 0.9904 Medium: 0.4793 High: 0.0138*	Control: 0.3592 Low: 0.3489 Medium: 0.8962 High: 0.9333
Day 7			Control: 0.8101 Low: 0.3547 Medium: 5617 High: 0.0162*
Day 14			

[a]Asterisk (*) indicates significant difference (*p* value ≤ 0.05).

Table 5.9. *p* values for carbonic anhydrase response between treatments following bicarbonate-only exposures[a].

Bicarbonate	Day 3	Day 7	Day 14
Day 3		Control: 0.0066* Low: 0.4662 Medium: 0.1694 High: 0.0069*	Control: 0.1384 Low: 0.8416 Medium: 0.3613 High: 0.6175
Day 7			Control: 0.1168 Low: 0.3575 Medium: 0.6172 High: 0.0179*
Day 14			

[a]Asterisk (*) indicates significant difference (*p* value ≤ 0.05).

Table 5.10. *p* values for carbonic anhydrase response between treatments following sulfate:bicarbonate mixture exposures[a].

Sulfate:Bicarbonate	Day 3	Day 7	Day 14
Day 3		Control: 0.2467 Low:High: 0.0046* Medium:Medium: 0.0435* High:Low: 0.1374	Control: 0.2550 Low:High: 0.0049* Medium:Medium: 0.9889 High:Low: 0.4558
Day 7			Control: 0.0327* Low:High: 0.9689 Medium:Medium: 0.0424* High:Low: 0.4276
Day 14			

[a]Asterisk (*) indicates significant difference (*p* value ≤ 0.05).

Table 5.11. *p* values for carbonic anhydrase response between treatments following chloride:bicarbonate mixture exposures[a].

Chloride:Bicarbonate	Day 3	Day 7	Day 14
Day 3		Control: 0.0650 Low:High: 0.5169 Medium:Medium: 0.3346 High:Low: 0.1552	Control: 0.0880 Low:High: 0.2411 Medium:Medium: 0.6657 High:Low: 0.0154*
Day 7			Control: 0.8649 Low:High: 0.0816 Medium:Medium: 0.5842 High:Low: 0.2168
Day 14			

[a]Asterisk (*) indicates significant difference (*p* value ≤ 0.05).

Table 5.12. *p* values for carbonic anhydrase response between treatments following chloride:sulfate mixture exposures[a].

Chloride:Sulfate	Day 3	Day 7	Day 14
Day 3		Control: 0.2208 Low:High: 0.6082 Medium:Medium: 0.6050 High:Low: 0.1264	Control: 0.0712 Low:High: 0.0205* Medium:Medium: 0.2193 High:Low: 0.5242
Day 7			Control: 0.0067* Low:High: 0.0077* Medium:Medium: 0.0926 High:Low: 0.3434
Day 14			

[a]Asterisk (*) indicates significant difference (*p* value ≤ 0.05).

Table 5.13. *p* values for changes in total ATPase activity following chloride-only exposures[a].

Chloride-Only	Control	Low	Medium	High
Control		Day 3: 0.5340 Day 7: 0.8248 Day 14: 0.1199	Day 3: 0.2562 Day 7: 0.0240* Day 14: 0.1582	Day 3: 0.9403 Day 7: 0.0345* Day 14: 0.3670
Low			Day 3: 0.0886 Day 7: 0.0375* Day 14: 0.8730	Day 3: 0.3869 Day 7: 00534 Day 14: 0.4852
Medium				Day 3: 0.2870 Day 7: 0.8571 Day 14: 0.5886
High				

[a]Asterisk (*) indicates significant difference (*p* value ≤ 0.05).

Table 5.14. *p* values for changes in total ATPase activity following sulfate-only exposures[a].

Sulfate-Only	Control	Low	Medium	High
Control		Day 3: 0.8625 Day 7: 0.5348 Day 14: 0.6959	Day 3: 0.7879 Day 7: 0.6851 Day 14: 0.4557	Day 3: 0.5086 Day 7: 0.1198 Day 14: 0.6372
Low			Day 3: 0.6590 Day 7: 0.8275 Day 14: 0.7188	Day 3: 0.6237 Day 7: 0.3280 Day 14: 0.3925
Medium				Day 3: 0.3563 Day 7: 0.2363 Day 14: 0.2309
High				

[a]Asterisk (*) indicates significant difference (p value ≤ 0.05).

Table 5.15 *p* values for changes in total ATPase activity following bicarbonate-only exposures[a].

Bicarbonate-Only	Control	Low	Medium	High
Control		Day 3: 0.9239 Day 7: 0.3692 Day 14: 0.9191	Day 3: 0.6708 Day 7: 0.8253 Day 14: 0.7740	Day 3: 0.7473 Day 7: 0.9220 Day 14: 0.7629
Low			Day 3: 0.6033 Day 7: 0.4942 Day 14: 0.8526	Day 3: 0.8204 Day 7: 0.3213 Day 14: 0.8411
Medium				Day 3: 0.4578 Day 7: 0.7503 Day 14: 0.9883
High				

[a]Asterisk (*) indicates significant difference (*p* value ≤ 0.05).

Table 5.16. *p* values for changes in total ATPase activity following sulfate:bicarbonate mixture exposures[a].

Sulfate:Bicarbonate	Control	Low:High	Medium:Medium	High:Low
Control		Day 3: 0.7986 Day 7: 0.5218 Day 14: 0.8824	Day 3: 0.7453 Day 7: 0.9134 Day 14: 0.6767	Day 3: 0.5684 Day 7: 0.4981 Day 14: 0.4849
Low:High			Day 3: 0.9442 Day 7: 0.5937 Day 14: 0.5733	Day 3: 0.4123 Day 7: 0.9699 Day 14: 0.5801
Medium:Medium				Day 3: 0.3748 Day 7: 0.5682 Day 14: 0.2712
High:Low				

[a]Asterisk (*) indicates significant difference (p value ≤ 0.05).

Table 5.17. *p* values for changes in total ATPase activity following chloride:bicarbonate mixture exposures[a].

Chloride:Bicarbonate	Control	Low:High	Medium:Medium	High:Low
Control		Day 3: 0.0666 Day 7: 0.5831 Day 14: 0.5187	Day 3: 0.4956 Day 7: 0.3739 Day 14: 0.6311	Day 3: 0.6468 Day 7: 0.7262 Day 14: 0.3788
Low:High			Day 3: 0.0169* Day 7: 0.1596 Day 14: 0.2673	Day 3: 0.0271* Day 7: 0.8411 Day 14: 0.8094
Medium:Medium				Day 3: 0.8193 Day 7: 0.2218 Day 14: 0.1822
High:Low				

[a]Asterisk (*) indicates significant difference (p value ≤ 0.05).

Table 5.18. *p* values for changes in total ATPase activity following chloride:sulfate mixture exposures[a].

Chloride:Sulfate	Control	Low:High	Medium:Medium	High:Low
Control		Day 3: 0.5435 Day 7: 0.9901 Day 14: 0.5529	Day 3: 0.0066* Day 7: 0.0178* Day 14: 0.0907	Day 3: <0.0001* Day 7: 0.0003* Day 14: 0.0099*
Low:High			Day 3: 0.0239* Day 7: 0.0182* Day 14: 0.0285*	Day 3: <0.0001* Day 7: 0.0003* Day 14: 0.0028*
Medium:Medium				Day 3: 0.0014* Day 7: 0.0720 Day 14: 0.2767
High:Low				

[a]Asterisk (*) indicates significant difference (*p* value ≤ 0.05).

Table 5.19. *p* values for total ATPase response between treatments following chloride-only exposures[a].

Chloride	Day 3	Day 7	Day 14
Day 3		Control: 0.0700 Low: 0.9237 Medium: 0.1545 High: 0.1405	Control: 0.0001* Low: 0.0057* Medium: 0.1060 High: 0.1261
Day 7			Control: 0.0039* Low: 0.0047* Medium: 0.8235 High: 0.9486
Day 14			

[a]Asterisk (*) indicates significant difference (*p* value ≤ 0.05).

Table 5.20. *p* values for total ATPase response between treatments following sulfate-only exposures[a].

Sulfate	Day 3	Day 7	Day 14
Day 3		Control: 0.7277 Low: 0.9402 Medium: 0.5012 High: 0.8382	Control: 0.0901 Low: 0.4283 Medium: 0.1682 High: 0.5145
Day 7			Control: 0.0487* Low: 0.4717 Medium: 0.4545 High: 0.6516
Day 14			

[a]Asterisk (*) indicates significant difference (*p* value ≤ 0.05).

Table 5.21. *p* values for total ATPase response between treatments following bicarbonate-only exposures[a].

Bicarbonate	Day 3	Day 7	Day 14
Day 3		Control: 0.8942 Low: 0.4098 Medium: 0.6644 High: 0.5781	Control: 0.7479 Low: 0.8885 Medium: 0.7012 High: 0.7502
Day 7			Control: 0.6504 Low: 0.4908 Medium: 0.9596 High: 0.8099
Day 14			

[a]Asterisk (*) indicates significant difference (*p* value ≤ 0.05).

Table 5.22. *p* values for total ATPase response between treatments following sulfate:bicarbonate mixture exposures[a].

Sulfate:Bicarbonate	Day 3	Day 7	Day 14
Day 3		Control: 0.5913 Low:High: 0.4468 Medium:Medium: 0.9322 High:Low: 0.9795	Control: 0.5281 Low:High: 0.7952 Medium:Medium: 0.3915 High:Low: 0.8530
Day 7			Control: 0.9235 Low:High: 0.3135 Medium:Medium: 0.3484 High:Low: 0.8731
Day 14			

[a]Asterisk (*) indicates significant difference (*p* value ≤ 0.05).

Table 5.23. *p* values for total ATPase response between treatments following chloride:bicarbonate exposures[a].

Chloride:Bicarbonate	Day 3	Day 7	Day 14
Day 3		Control: 0.9791 Low:High: 0.1504 Medium:Medium: 0.5279 High:Low: 0.5102	Control: 0.3961 Low:High: 0.0087* Medium:Medium: 0.3693 High:Low: 0.9078
Day 7			Control: 0.4102 Low:High: 0.1367 Medium:Medium: 0.7822 High:Low: 0.5855
Day 14			

[a]Asterisk (*) indicates significant difference (*p* value ≤ 0.05).

Table 5.24. *p* values for total ATPase response between treatments following chloride:sulfate exposures[a].

Chloride:Sulfate	Day 3	Day 7	Day 14
Day 3		Control: 0.3157 Low:High: 0.3231 Medium:Medium: 0.8271 High:Low: 0.2828	Control: 0.3711 Low:High: 0.9347 Medium:Medium: 0.2274 High:Low: 0.0885
Day 7			Control: 0.0716 Low:High: 0.3625 Medium:Medium: 0.3148 High:Low: 0.4799
Day 14			

[a]Asterisk (*) indicates significant difference (*p* value ≤ 0.05).

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CHAPTER SIX

CONCLUSIONS

The chronic toxicity of major ions and ion binary mixtures to *Ceriodaphnia dubia*

1. Conductivity is not a useful predictor of *C. dubia* chronic ion toxicity.
2. The relative order of toxicity for single ions resulting in a 50% decrease in reproduction of *C. dubia* was $Ca^{2+} \geq Mg^{2+} \geq SO_4^{2-} > HCO_3^- > Cl^- > Na^+$.
3. As single constituents, divalent ions had the largest impact on *C. dubia* reproduction.
4. The toxicity of magnesium is reduced with the addition of calcium.
5. The toxicity of bicarbonate is reduced with either addition of sulfate or chloride.
6. The chronic toxicity of single ions and ion binary mixtures are different from that previously described for acute ion toxicity.

The chronic toxicity of major ions and ion binary mixtures to *Pimephales promelas* (fathead minnow)

1. Conductivity is not a useful predictor of *P. promelas* chronic ion toxicity.
2. Effects on growth in *P. promelas*, described by a 50% reduction, demonstrated a relative order of toxicity for single ions as $SO_4^{2-} \geq Mg^{2+} > HCO_3^- \geq Ca^{2+} > Cl^- > Na^+$.
3. For the most part, divalent ions produced the greatest negative effect on *P. promelas* growth.
4. LC_{50} values were not significantly different from EC_{50} values estimated for sulfate, bicarbonate, magnesium, and calcium.
5. The addition of calcium reduced magnesium toxicity.

6. Chloride toxicity was reduced with the addition of sulfate.

The chronic toxicity of single ions and binary ion mixtures of an invertebrate species (*C. dubia*) and a vertebrate species (*P. promelas*): A comparison

1. *C. dubia* reproduction was effected at much lower dissolved ion concentrations than *P. promelas* growth, indicating that the invertebrate species was more sensitive than the vertebrate species.
2. Both species respond in a similar manner to changes in chloride, calcium, magnesium, and sodium concentrations, determined by similar concentration-response slopes.
3. For some ions and ion combinations, the effect on *P. promelas* growth may be a useful predictor of the effect on *C. dubia* reproduction.

The effect of multi-ion exposures on Na^+/K^+-ATPase and carbonic anhydrase activity and recovery in *Pimephales promelas* (fathead minnow)

1. *P. promelas* Na^+/K^+-ATPase was insensitive to digitalis compounds including ouabain and digoxin.
2. Gill carbonic anhydrase activity is significantly reduced by sodium bicarbonate, and does not recover 7-days after the exposure.
3. By day 7, total ATPase activity in the gill significantly increased following exposure to sodium chloride.
4. Sodium sulfate did not affect carbonic anhydrase or total ATPase activity within gill tissue of *P. promelas*.

5. A significant decrease in gill carbonic anhydrase and total ATPase activity was associated with mixtures containing high chloride and low sulfate concentrations.
6. Alterations in enzymatic activity in the gill of *P. promelas* following elevated anion exposures may indicate changes in energy allocation although at different energetic costs.

Dissolved ions, which contribute greatly to salinity, have become elevated in certain freshwater systems due to anthropogenic activities. Developing water quality criteria from inclusive measurements such as conductivity, have been attempted. However, combing all dissolved ions into one parameter assumes that they produce the same toxic response, and therefore, have similar effects on biological systems. Many studies, however, have suggested that conductivity is limited in its application, and is unsuitable for describing the toxicity of dissolved ions. The acute toxicity of elevated dissolved ions has been extensively investigated on many freshwater organisms. The implications of chronic exposures to these dissolved ions, however, is less understood. The overall goal of this research was to increase our understanding of the sub-lethal effects that result from chronic exposures of elevated dissolved ions to freshwater organisms, as well as investigate the mechanisms by which these dissolved ions produce their toxic effect.

The results of the present study indicate that increases in divalent ions, as individual components, produce the greatest response in both an invertebrate and vertebrate species. Additionally, although the invertebrate species was more sensitive to elevated dissolved ions, they responded similarly to changes in ion concentration on a

milli-molar basis as a vertebrate fish. These results suggest that *P. promelas* growth may be a useful predictor of *C. dubia* reproduction in response to elevated dissolved ions. Furthermore, these results demonstrate that the chronic toxicity of ion mixtures is different from those previously described for acute ion toxicity. These differences emphasize the need for additional chronic toxicity testing in order to fully understand the ion interactions leading to these detrimental responses. Although chronic toxicity responses are not initially observable such as those for acute toxicity, their effects should not be disregarded. Reproduction and growth are important functions that contribute to organismal fitness, and ultimately impact the success of an ecosystem. Incorporating these responses along with a physiologically based parameter regarding mechanisms of action into the development of predictive models, could aid in the establishment of national water quality criteria. With the myriad of anthropogenic sources of these ions, it is critical to develop reasonable watershed management strategies that also maintain a healthy aquatic ecosystem.